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ANNUAL PROGRESS REPORT FY 1971

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UNITED STATES ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES

Frederick, Maryland 21701

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13. ABSTRACT

A report of progress on the research program of the U. S. Army Medical Research Institute of Infectious Diseases on Medical Defense Aspects of Biological Agents (U) for Fiscal Year 1971 is presented.

KEYWORDS:

Biological warfare
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Therapy
Prophylaxis
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Bacterial diseases
Rickettsial diseases
Host
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Pathology
Defense
Metabolism
Virus diseases

U. S. ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES

FREDERICK, MARYLAND 21701

ANNUAL PROGRESS REPORT

FISCAL YEAR 1971

RCS-MEDDH-288(R1)

Project 1B662711A096 1 July 1971

SUMMARY

A report of progress on the research program of the U. S. Army Medical Research Institute of Infectious Diseases on Medical Defense Aspects of Biological Agents (U) for Fiscal Year 1971 is presented.

FOREWORD

This FY 1971 Annual Progress Report is a general review of research activities of the U. S. Army Medical Research Institute of Infectious Diseases, Frederick, Maryland, conducted under Project 1B662711A096, Medical Defense Aspects of Biological Agents (U) and a small effort under the In-house Laboratory Independent Research Program (ILIR). The project is divided into three tasks:

1B662711A096 01 - Pathogenesis of Infection of Military Importance.
1B662711A096 02 - Prevention and Treatment of Biological Agent Casualties.
1B662711A096 03 - Laboratory Identification of Biological Agents.

Nine contracts are currently in effect with educational institutions or industrial firms. Reports are available through DDC.

Tasks of the basic project are subdivided into work units, each identified by a three digit suffix. Numbers have been assigned in accordance with the following scheme:

General	001-099
Bacterial Diseases	100-299
Rickettsial Diseases	300-399
Viral Diseases	400-699
Mycotic Diseases	700-799
Intoxications	800-899
Contracts	900-999

Four appendices are included covering the Guest Lecture Series, Professional Staff Meetings, Formal Presentations and Briefings and a list of publications of the Institute for the Fiscal Year. An index by authors follows the main report.

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

1 July 1971

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PROJECT NO. 1B662711A096: MEDICAL DEFENSE ASPECTS OF BIOLOGICAL AGENTS (U)

* Terminated.

APPENDIXES

ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 01:	Pathogenesis of Infection of Military Importance
Work Unit No. 096 01 001:	Metabolic and Physiological Studies in Experimental Infectious Disease
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Division:	Physical Sciences
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19. RESPONSIBLE DOD ORGANIZATION
NAME:* USA Medical Research Institute of Infectious Diseases
ADDRESS:* Fort Detrick, Md 21701
RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
NAME:* Physical Sciences Division USAMRIID
ADDRESS:* Fort Detrick, Md 21701
PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME:* Bilbrey, G. L.
TELEPHONE: 301 663-4111 Ext 5214
SOCIAL SECURITY ACCOUNT NUMBER:
ASSOCIATE INVESTIGATORS
NAME: Walford, III, J. B.
NAME: Beisel, W. R. DA

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(U) Infections; (U) Physiology; (U) Renal function; (U) Salt and Water Metabolism; (U) Primates; (U) Acid-base balance; (U) Blood gases; (U) Lactate; (U) Military Medicine

23. TECHNICAL OBJECTIVE.* 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Study early changes in metabolism and physiology induced by experimental disease.

24 (U) A variety of techniques are employed to study metabolic changes associated with infection.

25 (U) 70 07 - 71 06 - I. Depression of free water clearance via the kidney occurs within 1 hr of pneumococcal bacteremia. Excessive water retention has been demonstrated in monkeys infected with Salmonella typhimurium studied over an 8-day period.

II. Normal values have been determined for caged and chair-restrained rhesus monkeys in the USAMRIID primate colony. It was found that both surgery and pneumococcal infection induced respiratory alkalosis. A variable rise in lactate levels was seen only postinfection. The mechanisms of the changes have not been determined as yet.

Publications: Proc. Amer. Soc. Nephrology 1970, p. 8.
Fed. Proc. 30:396, 1971.
J. Infect. Dis. 1971, In press.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 01: Pathogenesis of Infection of Military Importance

Work Unit No. 096 01 001: Metabolic and Physiological Studies in Experimental Infectious Diseases

Description:

Study early changes in metabolism and physiology induced by experimental disease.

Progress, Part I:

Alteration of renal function during systemic infectious illnesses in man has been evaluated in a retrospective fashion. A depression of glomerular filtration rate (GFR) has been demonstrated in cholera even after adequate fluid replacement[1/] (Amer. J. Med. 35:58, 1963). Normal GFR, as measured by creatinine clearance, was demonstrated in most patients with malaria, even in severe cases[2,3/] (Amer. J. Med. 43:735,745, 1967). Renal function declines in parallel with mean arterial blood pressure in staphylococcal sepsis[4/] (Ann. Surg. 162:161, 1965). Numerous studies have demonstrated the decline of urinary Na excretion and a fall in serum Na concentration occurring once certain infections are clinically apparent[5,6/] (J. Clin. Invest. 5:229, 1928; Amer. J. Dis. Child. 107:476, 1964).

Previous studies at this Institute have surveyed the sequential changes in urinary volume and electrolyte excretion which occur in volunteers inoculated with sandfly fever virus[7/] (Ann. Intern. Med. 67:744, 1967). Urinary electrolyte excretion was markedly decreased by the 3rd day after inoculation, and appeared to be related to decreased dietary intake. These studies did not contain specific measurements of renal function.

Concentrating defects have also been described. Free water reabsorption induced with vasopressin during human staphylococcal pneumonia[8/] (Metabolism 11:1181, 1962). Also, renal concentration defects have been demonstrated in the rat during several types of infection[9/] (J. Clin. Invest. 49:1427, 1970) and were thought to be directly related to the degree of renal invasion by the infecting organism.

Thus, both animal and human studies have shown changes in renal filtration and concentrating ability and urinary excretion of water and electrolytes during infection.

The work to be described concerns prospective studies performed in rhesus monkeys with blood borne bacterial infections.

Renal Function Studies--Gross parameters of renal function were measured in 12 conscious chaired monkeys. Following volume expansion with hypotonic saline, 6 monkeys were inoculated intravenously (IV) with virulent *Diplococcus pneumoniae*; 6 control monkeys were sham-inoculated with saline. In the bacteremic group, GFR remained stable and effective renal plasma flow (ERPF) showed a mild rise. Free water clearance (C_{H_2O}) became markedly depressed ($p < .01$) within 1 hr of inoculation and remained depressed for several hours; this depression was shown to be independent of GFR, plasma osmolality, temperature or changes in extracellular volume (ECV); it was thought to be mediated by infection-stimulated antidiuretic hormone (ADH) release.

Body Composition Studies--Seven conscious chaired monkeys were studied before and after IV inoculation with 1.0×10^8 *Salmonella typhimurium* organisms over a 9-day period. After inoculatin, GFR and ERPF declined significantly. Cardiac index increased but stroke volume remained unchanged. Monkeys became edematous and body weight increased approximately 10% from control levels despite a dietary intake below normal. Plasma volume and ECV rose significantly but could not entirely account for the increase in body weight. Total body water studies are incomplete at this time. Serial muscle biopsy studies were also performed but results are not yet available. Total exchangeable K was measured in 3 monkeys with no significant change from control levels. These data are being compared with control non-infected monkeys studied in an identical fashion before drawing final conclusions.

Summary, Part I:

Depression of free water clearance via the kidney occurs within 1 hr of pneumococcal bacteremia. Excessive water retention has been demonstrated in monkeys infected with *S. typhimurium* studied over an 8-day period.

Progress, Part II:

Many authors have reported normal values for blood gas-acid base balance in rhesus monkeys[10-13/] (Radiat. Res. 6:430, 1957; Temas de Medicina. p. 61, 1965; Proc. Soc. Exp. Biol. Med. 128:1183, 1968; J. Appl. Physiol. 28:108, 1970). However, no attempt has been made to (a) sample a large number of monkeys, (b) study sequential changes in blood gas-acid base balance, (c) study the response to surgery or (d) study the effects of infection. In addition, normal lactate values have not been established for the rhesus monkey.

The purpose, then, for this study is to correct these deficiencies.

Rhesus monkeys of both sexes, weighing 2.2 - 5.0 kg, were used. Blood samples were taken percutaneously from a femoral artery (in noncatheterized monkeys) into a heparinized glass syringe. The monkeys were given phencyclidine HCl (1.5 mg/kg) and atropine sulfate for the sampling procedures.

Blood for lactate determinations was immediately deproteinated in ice cold 10% tricholoroacetic acid, centrifuged, and the supernatant fluid was stored in a CO_2 freezer until the determinations could be made.

pH, pCO_2 and pO_2 values were immediately determined on the remaining blood sample using an Instrumentation Laboratories Model 113 pH/blood gas analyzer. Hemoglobin, temperature and white cell count (WBC) and differential were also monitored. pH was corrected for temperature and bicarbonate concentration ($[HCO_3^-]$) was determined using a nomogram[14] (Scand. J. Clin. Lab. Invest. 15 (Suppl 70), 1963).

Effects of surgery -- Early attempts to determine normal pH and blood gas values were accomplished using monkeys in restraint chairs; arterial catheters and thermocouples (for temperature measurement) had been installed surgically. It was noted that the catheterization procedure alone caused a rise in pH and drop in pCO_2. These values returned to normal or near normal (relative to published normal values[10-13])but did so only after a period of 10-15 days; some never returned to normal. This same phenomenon was seen after surgery in monkeys whose normal blood values were known. In addition, some animals seemed to "do poorly" after catheterization. Thus, to save time and animals, it was decided to perform all future studies using noncatheterized monkeys.

Normal values -- Normal values for pH, pCO_2,pO_2, $[HCO_3^-]$, and lactate were determined on 30 caged monkeys from the Animal Resources (USAMRIID) primate colony and on 16 monkeys from the same source that had been sitting in restraint chairs for 2 - 10 days. [The number of monkeys in the chair-restrained group will continue to grow as this study proceeds]. Normal values for caged monkeys are shown in Table I and those for chair-restrained monkeys in Table II (each value is the mean $\pm$ SD). As can be seen, the values for these 4 parameters agree closely. pCO_2 and $[HCO_3^-]$ are only slightly lower in the caged group.

TABLE I. NORMAL VALUES FOR 30 CAGED RHESUS MONKEYS.

PARAMETER	HOUR OF DAY	NUMBER OF SAMPLES	MEAN ± SD	RANGE
pH	0800	25	7.41 ± 0.06	7.223 - 7.514
	1000	26	7.40 ± 0.08	7.157 - 7.525
	1400	27	7.41 ± 0.07	7.174 - 7.530
	1600	27	7.42 ± 0.06	7.238 - 7.526
	1900	27	7.46 ± 0.07	7.239 - 7.596
	2100	28	7.46 ± 0.04	7.366 - 7.572
		160	7.43 ± 0.07	7.157 - 7.596
pCO_2 mm Hg	0800	25	27.4 ± 4.0	20.8 - 36.0
	1000	26	28.8 ± 3.9	21.2 - 34.9
	1400	27	29.4 ± 2.8	24.2 - 33.5
	1600	27	27.5 ± 2.8	22.8 - 32.6
	1900	27	29.8 ± 5.2	21.1 - 37.9
	2100	28	31.5 ± 5.1	20.3 - 39.9
		160	29.1 ± 4.3	20.3 - 39.9
pO_2 mm Hg	0800	25	73.9 ± 5.7	63.0 - 85.5
	1000	26	68.0 ± 5.8	60.5 - 78.5
	1400	27	76.8 ± 9.9	61.0 - 94.0
	1600	27	75.6 ± 6.7	64.0 - 90.5
	1900	27	76.0 ± 7.3	65.5 - 94.0
	2100	28	74.9 ± 7.3	60.0 - 91.0
		160	74.2 ± 7.7	60.0 - 94.0
$[HCO_3^-]$ mEq/L	0800	25	16.9 ± 3.6	7.9 - 23.0
	1000	26	17.6 ± 3.7	10.0 - 22.9
	1400	27	18.3 ± 3.4	11.0 - 25.0
	1600	27	17.3 ± 3.1	10.4 - 23.5
	1900	27	20.6 ± 5.3	8.8 - 31.9
	2100	28	22.3 ± 4.0	14.3 - 31.6
		160	18.9 ± 4.3	7.9 - 31.9
Lactate	0800	24	3.5 ± 2.4	0.5 - 8.0
	1000	25	4.5 ± 3.5	0.8 - 13.8
	1400	27	3.2 ± 2.8	0.6 - 9.1
	1600	27	3.9 ± 2.9	0.9 - 11.2
	1900	27	2.7 ± 2.4	0.6 - 9.8
	2100	28	2.5 ± 1.4	0.7 - 5.8
		158	3.4 ± 2.7	0.5 - 13.8

TABLE II. NORMAL VALUES FOR 16 CHAIRED, RESTRAINED RHESUS MONKEYS

PARAMETER	HOUR OF DAY	NUMBER OF SAMPLES	NUMBER OF MONKEYS	MEAN $\pm$ SD	RANGE
pH	0800	51	13	7.41 $\pm$ 0.06	7.241 - 7.519
	1000	37	12	7.44 $\pm$ 0.06	7.341 - 7.566
	1400	69	16	7.43 $\pm$ 0.04	7.333 - 7.547
	1500	20	7	7.43 $\pm$ 0.05	7.349 - 7.564
		177		7.42 $\pm$ 0.05	7.241 - 7.566
pCO_2	0800	51	13	32.7 $\pm$ 3.1	24.9 - 41.2
	1000	37	12	33.5 $\pm$ 5.4	24.4 - 43.8
mm Hg	1400	69	16	33.5 $\pm$ 4.9	24.5 - 46.0
	1500	20	7	31.7 $\pm$ 4.8	25.2 - 39.7
		177		33.1 $\pm$ 4.6	24.4 - 46.0
pO_2[a/]	0800	45	13	75.6 $\pm$ 9.3	60.0 - 99.5
	1000	35	10	75.7 $\pm$ 5.9	62.5 - 89.0
mm Hg	1400	67	15	76.0 $\pm$ 7.8	61.0 - 95.5
	1500	18	8	75.1 $\pm$ 7.4	62.5 - 90.5
		165		75.7 $\pm$ 7.8	60.0 - 99.5
$[HCO_3^-]$	0800	51	13	20.0 $\pm$ 3.7	10.4 - 29.5
	1000	37	12	22.1 $\pm$ 5.1	14.1 - 31.6
mEq/L	1400	69	16	21.8 $\pm$ 4.2	14.2 - 33.7
	1500	20	7	20.7 $\pm$ 4.3	15.4 - 28.7
		177		21.3 $\pm$ 4.3	10.4 - 33.7
Lactate	0800	42	13	2.0 $\pm$ 1.2	0.3 - 8.3
	1000	34	10	1.6 $\pm$ 1.7	0.4 - 8.9
mEq/L	1400	59	16	1.5 $\pm$ 1.0	0.4 - 5.6
	1500	17	7	1.9 $\pm$ 1.3	0.6 - 4.8
		152		1.7 $\pm$ 1.4	0.3 - 8.9

a. 17 animals in the group

Values on individual monkeys varied considerably over different time periods on different days (Table III). However, the average value for 30 monkeys for 6 time periods (0800, 1000, 1400, 1600, 1900 and 2100 hours) and for 16 monkeys for 4 time periods (0800, 1000, 1400, 1500 hours) showed no significant variation. This indicates that although individual monkeys vary in their hourly and daily levels, this variation does not form a cyclic pattern.

The larger difference noted in mean lactate levels between the 2 groups is possibly due to the difference in activity. Caged animals were still free to roam about the cage, while chair restrained monkeys tended to sit quietly throughout the day. This difference in muscular work would be expected to result in a higher lactate level in caged monkeys. None of the monkeys were hypoxic, and none showed evidence of frank disease so these causes of hyperlactatemia can probably be discarded.

In both caged and restrained monkeys, values for pH agreed well with previously published values for the rhesus monkey. Values for pCO_2, $[HCO_3^-]$ and pO_2 agreed with some published data for the rhesus, but are somewhat lower than corresponding human values (Table IV). Blood lactate levels in monkeys appear to be higher than in humans at rest.

The higher lactate levels in monkeys can probably be explained on the basis of increased exercise relative to resting humans, but the low pCO_2 and $[HCO_3^-]$ levels found in both groups are harder to explain. These may be the result of unknown prior conditions which have caused a loss of total CO_2, or may reflect the fact that monkeys have lower levels for pCO_2 and $[HCO_3^-]$. The latter possibility seems unlikely.

It is interesting to note that variation in individual parameters as reflected in their standard deviations was equal to or less than the variation in those same parameters as measured by most other investigators. This may be due to the larger number of monkeys used in this study.

Effects of Infection -- In chaired, noncatheterized rhesus monkeys infected IV with D. pneumoniae resulted in a variable rise in pH, a drop in pCO_2 and a drop in $[HCO_3^-]$. The rise in pH coupled with a drop in pCO_2 indicates the presence of respiratory alkalosis. However, this relationship does not have a quantitative correlation. The drop in $[HCO_3^-]$ may be due to: (a) a loss of total CO_2 due to "blowing off" of CO_2 or (b) a response to some metabolic change which is reflected in the $[HCO_3^-]$ level. Although (a) is favored by this investigator on a subjective basis, neither cause can be ruled out.

Blood lactate values were variably increased after D. pneumoniae infection. The rise varied from 165% to 580% of the individual's control values; this large variability is reflected in the large standard deviations given for each time period. The rise did not have a quantitative correlation with any other acid-base parameter although its occurrence corresponded to the changes in pH, pCO_2 and $[HCO_3^-]$. The rise in

TABLE III. VALUES FOR SELECTED MONKEYS SHOWING THE VARIATION POSSIBLE IN BLOOD GAS-ACID BASE PARAMETERS

MONKEYS Condition	(#)	Hour	pH	pCO_2 mmHg	pO_2 mmHg	$[HCO_3^-]$ mEq/L	Lactate mEq/L
Chaired	(547)	0800	7.36	30.9	78.0	16.8	1.2
		1000	7.34	37.3	80.5	19.6	1.[illegible]
		1400	7.38	39.4	84.5	22.7	1.3
		1500	7.40	39.4	73.0	23.3	0.8
	(610)	0800	7.46	31.7	86.5	21.5	0.9
		1000	7.37	42.8	78.0	23.0	0.9
		1400	7.49	28.2	85.0	21.0	2.1
		1500	7.48	39.3	79.5	28.7	----
Caged	(788)	0800	7.22	20.3	82.5	7.9	5.3
		1000	7.46	24.3	77.5	16.9	2.2
		1600	7.41	27.8	80.0	17.0	5.9
		2100	7.47	28.3	81.0	20.4	1.6
	(804)	0800	7.41	32.2	72.5	19.6	6.7
		1000	7.39	34.4	62.5	20.4	5.8
		1400	7.49	33.5	80.0	25.0	1.2
		1600	7.40	28.4	67.0	16.7	7.6
		1900	7.39	36.1	72.5	21.0	1.1
		2100	7.45	39.0	81.0	26.1	2.9

TABLE IV. NORMAL VALUES FOR MONKEYS COMPARED TO DATA FROM OTHER INVESTIGATORS (MEAN ± SD)

SOURCE: (no of species)	pH	pCO_2 mm Hg	pO_2 mm Hg	$[HCO_3^-]$ mEq/L	LACTATE mEq/L
RHESUS					
Present Study					
Chaired (30)	7.42 ± 0.05	33.1 ± 4.6	75.7 ± 7.8	21.3 ± 4.3	1.7 ± 1.4
Caged (16)	7.43 ± 0.07	29.1 ± 4.3	74.2 ± 7.7	18.9 ± 4.3	3.4 ± 2.7
Ref. 11 (14)	7.43 ± 0.07	42.9 ± 8.5		26.7 ± 4.25[a]	
Ref. 13 (6)	7.46 ± 0.04	42.6 ± 1.0	100.8 ± 7.0		
Ref. 10 (4)		31	65		
CYNOMOLGUS					
Ref. 12 (45)	7.25 ± 0.08	28 ± 5	109 ± 10		
Ref. 13 (6)	7.47 ± 0.02	38.8 ± 2.7	100.7 ± 4.2		
HUMAN[15]	7.35 - 7.45	35 - 45	75 - 100	24	0.6 - 1.8

a. Standard HCO_3^-.

TABLE V. EFFECT OF D. PNEUMONIAE INFECTION ON pH, pCO_2, HCO_3^- AND LACTATE OF MONKEYS

TIME DAY	am pm	% OF CONTROL (100%) pH Infected	pH Controls	pCO_2 Infected	pCO_2 Controls
0	pm	99.7 ± 0.6	100.1 ± 0.5	103.8 ± 21.9	97.2 ± 22.9
1	am	100.1 ± 0.0	99.7 ± 0.3	75.3 ± 10.3	91.8 ± 8.6
	pm	100.5 ± 0.6	99.9 ± 0.7	70.2 ± 13.0	85.3 ± 10.2
2	am	100.1 ± 0.7	99.7 ± 0.6	72.5 ± 12.9	93.6 ± 10.6
	pm	100.8 ± 0.6	99.9 ± 1.0	65.5 ± 15.5	89.3 ± 11.2
3	am	100.8 ± 0.7	100.3 ± 0.7	77.1 ± 14.4	97.3 ± 14.1
	pm	100.8 ± 0.8	100.3 ± 0.7	70.6 ± 11.4	92.9 ± 9.1
4	am		100.0 ± 0.9	89.6 ± 24.1	110.0 ± 23.8
	pm	100.7 ± 1.4	100.5 ± 1.8	81.2 ± 17.0	96.4 ± 19.2

TIME DAY	am pm	$[HCO_3^-]$ Infected	$[HCO_3^-]$ Controls	Lactate Infected	Lactate Controls
0	pm	99.2 ± 20.1	96.1 ± 18.2	157.8 ± 55.8	152.4 ± 163.7
1	am	73.1 ± 13.8	85.6 ± 13.0	96.6 ± 42.3	86.2 ± 83.8
	pm	76.1 ± 13.1	82.1 ± 16.7	177.7 ± 33.8	114.7 ± 18.5
2	am	74.4 ± 15.4	84.0 ± 8.3	323.4 ± 163.7	188.3 ± 141.1
	pm	76.4 ± 22.1	87.7 ± 28.7	185.5 ± 5.4	
3	am	89.5 ± 21.3	99.3 ± 23.2		
	pm	83.5 ± 23.9	94.5 ± 23.4	160.9 ± 93.7	79.9 ± 20.2
4	am		112.5 ± 40.7		
	pm	96.1 ± 34.6	112.6 ± 49.4	181.6 ± 61.2	161.8 ± 140.8

blood lactate also corresponded to postinfection swelling and edema of the legs and feet of the infected monkeys. Although this appears to be the closest to a quantitative correlation when viewed subjectively, no objective measurements have been made. Thus the cause of the rise of lactate values postinfection is still undetermined.

Since this study is not yet completed, no attempt has been made to apply statistical analysis to the data. Future work will include an attempt to elucidate the causes of these acid base-blood gas changes. In addition the study will be expanded to include a gram negative infectious organism, e.g., Salmonella.

Summary, Part II:

Normal values for acid base-blood gas parameters have been determined for individuals in the USAMRIID primate colony. Values were determined for 6 time periods on caged rhesus monkeys and 4 time periods on chair-restrained monkeys. The overall average values for pH, pCO_2, pO_2, HCO_3^- and lactate did not differ for the 2 groups.

The procedure of arterial catheterization resulted in a respiratory alkalosis lasting 10-12 days or longer. Infection of rhesus monkeys with D. pneumoniae resulted in a respiratory alkalosis plus a variable rise in lactate levels. Mechanisms for these changes have not been determined.

Presentations:

1. Bellanti, J. A., M. C. Yang, R. I. Krasner, P. J. Bartelloni, and W. R. Beisel. Studies of leukocyte function in the human during experimental sandfly fever virus infection. Presented at Annual Meeting of the Federation of American Societies for Experimental Biology, Chicago, Ill. 12-17 April 1971.

Publications:

1. Bilbrey, G. L., and W. R. Beisel. Nov. 1970. Depression of free water clearance (C_{H_2O}) during pneumococcal bacteremia. Proc. Am. Soc. Nephrology, p.8 (abstract).

2. Bellanti, J. A., M. C. Yang, R. I. Krasner, P. J. Bartelloni, and W. R. Beisel. 1971. Studies of leukocyte function in the human during experimental sandfly fever virus infection. Fed. Proc. 30:396 (abstract).

3. Bilbrey, G. L., and W. R. Beisel. 1971. Depression of free water clearance during pneumococcal bacteremia. J. Infect. Dis. In press.

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12. Banerjee, C. M., Y. Alarie and M. Woolard. 1968. Gas tension in conscious monkeys. Proc. Soc. Exp. Biol. Med. 128:1183-1185.

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14. Siggaard-Andersen, O. 1963. The acid-base status of the blood. Scand. J. Clin. Lab. Invest. 15 (Suppl 70): 1-134.

15. Castleman, B. (ed.) 1970. Normal laboratory values. New Eng. J. Med. 283:1276-1285.

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ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 01:	Pathogenesis of Infection of Military Importance
Work Unit No. 096 01 002:	Role of Hormones in Infectious Disease
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Division:	Physical Sciences
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Author:	Frederick R. DeRubertis, Major, MC
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

16

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
	DA OL0801	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 01 06	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	01	002
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	CDOG 1212b(9)			

11. TITLE (Precede with Security Classification Code)
(U) Role of Hormones in Infectious Disease of Military Medical Importance (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
003500 Clinical Medicine; 002300 Biochemistry; 004900 Defense

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
62 07	CONT	DA	C. In-house

17. CONTRACT GRANT
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER: NA
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	1	60
	CURRENT 72	1	71

19. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, Md 21701
RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
NAME: Physical Sciences Division USAMRIID
ADDRESS: Fort Detrick, Md 21701
PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: DeRubertis, F. R.
TELEPHONE: 301 663-4111 Ext 5158
SOCIAL SECURITY ACCOUNT NUMBER:
ASSOCIATE INVESTIGATORS
NAME:
NAME: DA

21. GENERAL USE
Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Thyroid Hormones; (U) Kinetics; (U) Pneumococcus; (U) Catecholamines; (U) Insulin; (U) Encephalitis; (U) Sandfly fever; (U) Growth hormone; (U) Radioisotope (U) Military Medicine

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Study the role of hormones in host response to infection.

24 (U) Isotope tracer techniques are employed in assessing cellular uptake and subcellular distribution of hormones during acute infection.

25 (U) 70 07 - 71 06 - Evidence for enhanced cellular thyroxine binding was obtained in vivo during acute pneumococcal infection in the rhesus monkey. This phenomenon might contribute to the increased turnover of thyroxine previously reported and suggests early alteration in peripheral avidity for thyroxine as a mechanism initiating enhanced turnover and secretion.

Publication: Clin. Invest. 50:378-387, 1971.
Clin. Res. 19:371, 469, 1971.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 01 Pathogenesis of Infection of Military Importance

Work Unit No. 096 01 002: Role of Hormones in Infectious Disease

Description:

Study the role of hormones in host response to infection.

Progress:

Increased metabolic clearance of both L-thyroxine (T4) and L-triiodothyronine (T3) has been observed during acute pneumococcal infection in man[1] (J. Clin. Endocr. 27:93, 1967) and the rhesus monkey[2] (J. Clin. Invest. 50:378, 1971) accompanied by probable increased glandular hormonal secretion.[2] In the rhesus monkey the concurrent increased clearance of T4 and T3 and the absence of changes in the distribution of T4 among its major serum binding protein early in the course of this infection suggested an enhanced cellular binding of thyroid hormone.[2] The latter event might initiate the observed acceleration of peripheral hormonal clearance and the subsequent increase in glandular secretion. The present studies were conducted in an attempt to evaluate (a) cellular uptake and binding of T4 in vivo early in the course of acute pneumococcal infection, (b) to identify differences in infection, if any, in the uptake of hormone by the major binding organs (liver and kidney), (c) to identify differences if any, in the subcellular distribution of hormone, and (d) to assess whether the increased metabolic clearance of thyroid hormone which occurs during pneumococcal infection is accompanied by evidence of enhanced biologic action of hormone at the cellular level.

Studies were conducted in chaired male rhesus monkeys who after a period of adaptation were intravenously (IV) inoculated with either 10^8 viable or heat killed Diplococcus pneumoniae. Cellular uptake of thyroxine (CT4) was assessed using a double isotope (albumin - ^{125}I and T4 - ^{131}I) two-compartmental (albumin space and cellular space) kinetic model previously described.[3] (J. Clin. Invest. 46:762, 1967). At either 12 or 24 hr after inoculation with pneumococci monkeys were given 10 µCi each of albumin - ^{125}I and T4 - ^{131}I IV: blood samples were obtained at 10-min intervals for the next 60 min. CT4 was calculated from the difference in the instantaneous distribution volumes of the 2 isotopes. Hepatic and renal uptakes were estimated by whole organ counting and by a biopsy technique,[3] both methods yielding similar results. In more recent experiments hepatic subcellular distribution of T4-^{131}I was evaluated employing a standard cell separation procedure[4] (Biochem. J. 92:179, 1964) and appropriately

correcting serum contamination of each subcellular fraction[5/] (Endocrinology 84:270, 1969). To assess extracellular T4 protein binding, the proportion of free T4 (FT4) in serum was measured by equilibrium dialysis[6/] (J. Clin. Invest. 47:1710, 1968) and absolute serum free T4 values (AFT4) calculated from the product of total serum T4 concentration and percentage FT4.

CT4 calculated 60 min after isotope injection was significantly increased in monkeys studied 12 and 24 hr after infection. The increased CT4 in the infected monkeys was apparent within 10 min. Analysis of the albumin - ^{125}I and T4 - ^{131}I serum disappearance curves indicated that the increased CT4 in the control and infected monkeys was due to a more rapid disappearance of T4- ^{131}I in the infected population. The early appearance of this change made it unlikely that metabolism of hormone contributed importantly to the accelerated T4 disappearance.

Increased CT4 in the infected monkeys was not accounted for by either increased hepatic or renal uptake, which expressed as % administered dose of T4 - ^{131}I were not statistically different in control and infected groups. These results suggest that other cellular sites may be involved in the enhanced CT4 observed during this infection. However, with the currently employed techniques the specific sites involved could not be identified.

FT4 and AFT4 at 12 hr were not significantly different in control and infected monkeys. Thus in the infected monkeys the increased CT4 appeared to precede any detectable decrease in extracellular protein binding of T4, suggesting an increase in cellular binding of hormone early in this infection.

Only preliminary data has been obtained on the hepatic subcellular distribution of T4 - ^{131}I in pneumococcal infection in the rhesus. No marked changes have been observed. Initial experiments suggest and a small decrease in the proportion of labeled hormone found in the soluble fraction. However, no conclusions are warranted at present.

An attempt was made to determine whether the accelerated peripheral turnover of thyroid hormone during pneumococcal infection was associated with evidence of increased cellular biologic action of hormone by measuring the activity of hepatic mitochondrial α-glycerophosphate dehydrogenase (HMGPD). This enzyme is known to be quite readily and rapidly induced by thyroid hormone administration in the rat[7/] (J. Biol. Chem. 240:1427, 1965) and is known not to be induced by glucosteroids. It thus appeared to be an appropriate tool to employ in acute infection. The method of Ruegamer et al[8/] (Endocrinology 75:908, 1964) was used in assaying enzyme activity. In our hands increased activity of HMGPD in rats could be demonstrated with this method within 12 hr after a single parenteral dose

of T3. Unfortunately no increase in HMGPD could be demonstrated in the rhesus monkey even after prolonged administration of large doses of T4 and T3. Similarly no increase in activity of this enzyme was observed in monkeys examined 24 and 48 hr after IV pneumococcal infection. When, however, HMGPD activity was examined in rats 24 hr after pneumococcal infection a small increase was noted. This observation is being examined further.

Summary:

Evidence for enhanced cellular thyroxine binding was obtained *in vivo* during acute pneumococcal infection in the rhesus monkey. This phenomenon might contribute to the increased turnover of thyroxine previously reported and suggests early alteration in peripheral avidity for thyroxine as a mechanism initiating enhanced turnover and secretion.

Publications:

1. Woeber, K. A. 1971. Alterations in thyroid hormone economy during acute infection with *Diplococcus pneumoniae* in the rhesus monkey. J. Clin. Invest. 50:378-387.

2. DeRubertis, F. R., and K. A. Woeber. 1971. Evidence for enhanced cellular thyroxine binding in vivo in pneumococcal infection. Clin. Res. 19:371 (abstract).

3. Winnacker, J. L. 1971. Regulation of growth hormone secretion in acute infection. Clin. Res. 19:70 (abstract).

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alanine aminotransferase, D-amino acid oxidase and catalase in rat-liver tissue. Biochem. J. 92:179-184.

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ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 01:	Pathogenesis of Infection of Military Importance
Work Unit No. 096 01 003:	Tissue Enzyme Changes in Infectious Disease
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Division:	Physical Sciences
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Author:	Gordon L. Bilbrey, Major, MC
Reports Control Smybol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

32

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
	DA OL0802	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 01 06	D. CHANGE	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	01	003
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	CDOG 1212b(9)			

11. TITLE (Precede with Security Classification Code)*
(U) Tissue Enzyme Changes in Infectious Disease of Military Medical Importance (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*
002300 Biochemistry; 003500 Clinical Medicine; 004900 Defense

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
64 10	CONT	DA	C. In-house

17. CONTRACT GRANT
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER*:
c. TYPE: NA
d. AMOUNT:
e. KIND OF AWARD:
f. CUM. AMT.:

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	2	20
	CURRENT 72	0	12

19. RESPONSIBLE DOD ORGANIZATION
NAME* USA Medical Research Institute of Infectious Diseases
ADDRESS* Fort Detrick, Md 21701
RESPONSIBLE INDIVIDUAL
NAME Crozier, D.
TELEPHONE 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
NAME* Physical Sciences Division USAMRIID
ADDRESS* Fort Detrick, Md 21701
PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME* Bilbrey, G. L.
TELEPHONE 301 663-4111 Ext 5214
SOCIAL SECURITY ACCOUNT NUMBER
ASSOCIATE INVESTIGATORS
NAME:
NAME: DA

21. GENERAL USE
Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Pneumococcus; (U) Infection; (U) Enzyme induction; (U) Tyrosine transaminase; (U) Fetal; (U) Pregnancy; (U) Military Medicine

23. TECHNICAL OBJECTIVE.* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) To study serial changes in tissue enzyme systems during the course of experimental infections.

24 (U) Tryptophan tolerance tests are performed serially in individuals in whom infections have been experimentally induced, in order to study the activity of hepatic tryptophan pyrrolase. Urinary diazo reactants and kynurenine pathway metabolites are measured.

25 (U) 70 07 - 71 06 - The principal investigator for this work unit was separated from the Army. No further work has been carried out. If the condition is still the same in January 1972, the work unit will be terminated.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 01: Pathogenesis of Infection of Military Importance

Work Unit No. 096 01 003: Tissue Enzyme Changes in Infectious Disease

Description:

To study serial changes in tissue enzyme systems during the course of experimental infection.

Progress and Summary:

The principal investigator for this work unit was separated from the Army. No further work has been carried out. If the condition is still the same in January 1972, the work unit will be terminated.

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ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 01:	Pathogenesis of Infection of Military Importance
Work Unit No. 096 01 005:	Evaluation of Normal Colony Animals
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Divisions:	Animal Assessment, Pathology and Physical Sciences
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Authors:	Richard O. Spertzel, Lt Colonel, VC (I, II) Glen E. Marrs, Captain, VC (I, II) James L. Stookey, Lt Colonel, VC (I, III) Garrett S. Dill, Jr., Captain, VC (I, III) Gary L. Cockerell, Captain, VC (II) Robert S. Pekarek, Ph.D. (II)
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

26

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
	DA 0L0804	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 01 06	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	01	005
b. CONTRIBUTING				
c. ~~[illegible]~~	CDOG 1212b(9)			

11. TITLE (Precede with Security Classification Code)
(U) Evaluation of Normal Colony Animals for BW Defensive Research (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
002600 Biology; 003500 Clinical Medicine; 004900 Defense

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
64 08	CONT	DA	C. In-house

17. CONTRACT/GRANT
a. DATES/EFFECTIVE — EXPIRATION
b. NUMBER — NA
c. TYPE — d. AMOUNT
e. KIND OF AWARD — f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	3	40
	CURRENT 72	3	30

19. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, Md 21701

RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
NAME: Animal Assessment and Pathology Div USAMRIID
ADDRESS: Fort Detrick, Md 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Spertzel, R. O.
TELEPHONE: 301 663-4111 Ext 7244
SOCIAL SECURITY ACCOUNT NUMBER

ASSOCIATE INVESTIGATORS
NAME: Marrs, G. E.
NAME: Stookey, J. L.

21. GENERAL USE
Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Disease; (U) Normal values; (U) Tuberculin tests; (U) Blood culture; (U) Hematology; (U) Natural antibodies; (U) Gastroenteritis; (U) Shigella; (U) SEB; (U)Military Medicine

23. TECHNICAL OBJECTIVE. 24. APPROACH 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Obtain clinical and pathological baseline values. Establish patterns of disease in normal colony animals.

24 (U) Conduct studies on colony animals to establish normal values for various biological parameters of interest to investigators using animals as test subjects. Study incidence and patterns of disease in the animals.

25 (U) 70 07 - 71 06 - Animal Resources Section received 533 monkeys. More than 3,200 tuberculin tests were done of which only 1 was suspect, but no gross or histopathologic evidence of Mycobacterium tuberculosis was found at necropsy. Blood culture, hemoglobin and hematocrit determinations, white blood cell and differential counts were obtained on all animals issued from the Section. Sixteen per cent of the monkeys received had natural antibodies against staphylococcal enterotoxin B.

Gastroenteritis continued to be the most common disease problem in the colony; 165 monkeys were treated for diarrhea. Shigella spp. were isolated from 73.2% of the diarrhea cases in which cultures were attempted.

Publications: Lab. Anim. Care 20:681-685, 1970.
Lab. Anim. Sci., 1971, In press.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 01: Pathogenesis of Infection of Military Importance

Work Unit No. 096 01 005: Evaluation of Normal Colony Animals

Description:

Obtain baseline clinical values, search for pathological lesions, and establish patterns of disease in normal colony animals.

Progress, Part I:

Goats and burros were screened for parasitic infestations and given anti-helminthic drugs as indicated. Strongylosis was the most frequently occurring infestation encountered.

Burros were branded; goats were identified with neck chain and tag.

Monkeys - A total of 533 rhesus monkeys (*Macaca mulatta*) was received by Animal Resources Section (AR), 537 was the greatest number and 373 the smallest maintained at one time in the Section. Monkeys were received from Divisions of the U. S. Army Biological Defense Research Center, Fort Detrick, as follows: 366 from the Animal Farm, 50 from Pathology, 28 from Special Operations, and 89 from Medical Bacteriology. Of these, 16.7% had natural antibodies against staphylococcal enterotoxin B (SEB).

A total of 165 monkeys was treated for diarrhea, 127 of which were bloody. *Shigella* was isolated in 104 of 142 (73.2%) of the diarrhea cases from which cultures were obtained and identified as follows: 97 as *Sh. flexneri* 4; 5, *Sh. dysenteriae*; and 2, *Sh. sonnei*. Deaths occurred as follows: shigellosis--3 monkeys; unexpected--9 monkeys, 1 goat, 1 burro foal; euthanitized--7 monkeys 1 goat, and 1 adult burro.

In December 1970 one monkey with signs of severe muscle atrophy and apparent fibrosis was euthanitized and necropsied. The only significant histological finding was a severe infestation with Sarcosporidia and severe eosinophilic myositis.

It is evident that the isolation of sick monkeys in a hospital room in each suite and treatment for a minimum of 3 or 5 days after apparent recovery, whichever is longer, is helping to reduce the disease problem in relation to diarrhea.

Eighty monkeys were received from the Fort Detrick Animal Farm Division on 27 Aug 70. Upon receipt, 32 of the monkeys were placed on 25.0 mg tetracycline HCl intramuscularly (IM), twice a day for 5 days. In the first 30 days after they arrived, there was no diarrhea in the animals placed on pro-

phylactic tetracycline. During the same period, in the 48 monkeys not placed on prophylactic tetracycline therapy, there were 14 cases of diarrhea, one of which terminated in death. At the termination of the 30-day holding period, the 32 monkeys placed on the prophylactic tetracycline underwent a series of 10 rectal cultures in an attempt to determine if they were harboring Shigella organisms in a carrier state. Sh. flexneri 4 was isolated 1-4 times in 12 of these 32 monkeys. It was noted that during the second 30-day period, the group that had not been on tetracycline prophylaxis had relatively no diarrhea, while the group that had been on prophylaxis had an increased incidence of diarrhea.

Koch's Old Tuberculin (KOT) was used for 3223 intrapalpebral tests of monkeys; of which all but one were negative. The one suspect (Monkey No. A-627) was euthanitized. There was no evidence of infection with Mycobacterium tuberculosis by gross or microscopic examination of tissue.

AR is presently ordering and delivering feed and bedding for all divisions of the Institute.

Summary, Part I:

Gastroenteritis continued to be the most common disease problem. Prophylactic therapy with tetracycline HCl immediately after an animal was received by AR appears to decrease the diarrhea incidence for the first 30 days, although it appears to have no effect on the animal as a carrier of Shigella organisms. Due to the diarrhea in this group, it is felt that after the first 30 days, even though the diarrhea was reduced, the tetracycline acted merely as a delaying factor.

Progress, Part II:

Blood culture, hemoglobin and hematocrit determinations, and white blood cell and differential counts were done on all M. mulatta being issued from AR Section.

A study is in progress to determine baseline levels of the following parameters in conditioned M. mulatta: serum proteins and glycoproteins; serum protein-bound hexose, hexosamine, sialic acid and fucose; haptoglobin-types and quantities; and serum Zn, Fe and Cu. One hundred and seventy M. mulatta will be bled for 6 fasting blood samples; and quantitative procedures will be carried out by Physical Sciences Division.

Summary, Part II:

Blood cultures and hematology are being done routinely on monkeys leaving Bldg. 1425. A study is in progress to determine baseline levels for a number of serum levels of interest to the Institute.

Progress and Summary, Part III:

As previously discussed elsewhere in this report, 20 normal colony animals of various species were necropsied. These represent animals from the normal colony which either died spontaneously, or were euthanitized because of various illnesses or disorders. In addition to these animals, members of the Pathology Division necropsied and accessioned 678 animals of various species from other divisions in the Institute. These animals also represent normal colony animals, although they had been issued and subjected to experimental procedures.

The most significant accomplishment of the year, as far as the *M. mulatta* colony was concerned, was the absence of tuberculosis. The vigorous eradication program following last year's outbreak[1] (USAMRIID Annual Progress Report, FY 1970, p. 21) was apparently effective in eliminating this disease from the primate colony.

Except for the enteric disorders previously mentioned in the monkeys, the general health of all species of laboratory animals appears to be markedly improved. Many diseases previously described[1] were not observed this year.

Publications:

1. Rollins, J. B., C. H. Hobbs, R. O. Spertzel, and S. McConnell. 1970. Hematologic studies of the rhesus monkey (*Macaca mulatta*). Lab. Anim. Care 20:681-685.

2. Weil, Jerry D., M. K. Ward, and R. O. Spertzel. 1971. Incidence of *Shigellae* in conditioned rhesus monkeys (*Macaca mulatta*). Lab. Anim. Sci. In press.

LITERATURE CITED

1. U. S. Army Medical Research Institute of Infectious Diseases. 1 July 1970. Annual Progress Report, FY 1970. p. 21 to 29.

ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 01:	Pathogenesis of Infection of Military Importance
Work Unit No. 096 01 009:	Host Amino Acid, Protein and RNA Metabolism During Infectious Disease
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Division:	Physical Sciences
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Authors:	Robert W. Wannemacher, Jr., Ph.D., (I, II, III) William R. Beisel, M.D., (I) Peter J. Bartelloni, Lt. Colonel, MC, (I) Robert S. Pekarek, Ph.D., (II) Michael C. Powanda, Captain, MSC, (II)
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OL0879	71 07 01	DD DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 01 06	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	01	009
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	CDOG 1212b(9)			

11. TITLE (Precede with Security Classification Code) (U) Host Amino Acid, Protein and RNA Metabolism during Infectious Disease of Military Medical Importance (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
002300 Biochemistry; 003500 Clinical Medicine; 004900 Defense

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
65 07	CONT	DA	C. In-house

17. CONTRACT GRANT
a. DATES/EFFECTIVE — EXPIRATION
b. NUMBER
c. TYPE — NA — d. AMOUNT
e. KIND OF AWARD — f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	3	50
	CURRENT 72	3	94

19. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, Md 21701
RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
NAME: Physical Sciences Division USAMRIID
ADDRESS: Fort Detrick, Md 21701
PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Wannemacher, R. W.
TELEPHONE: 301 663-4111 Ext 6130
SOCIAL SECURITY ACCOUNT NUMBER
ASSOCIATE INVESTIGATORS
NAME: Beisel, W. R.
NAME: Vollmer, R. T. DA

21. GENERAL USE
Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Amino acids; (U) Sandfly fever; (U) Typhoid fever; (U) Adenovirus-21 infection; (U) Military Medicine

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Study changes in amino acids of blood and tissues in infectious disease or in conditions induced by other variables.

24 (U) Free amino acid concentrations are determined by ion-exchange chromatography in plasma and tissue of experimental subjects infected with bacterial or viral organisms.

25 (U) 70 07 - 71 06 - Studies to determine free amino acid changes during the course of infection with various microorganisms in man and laboratory animals have been continued; a mechanism for the mediation of the changes has been elucidated.

Both viral and bacterial infections in humans and nonhuman models are characterized by an early depression in serum free amino acids. The changes in individual amino acids appear to be characteristic of the infection under study. The decrease is related to a flow of amino acids from muscle and liver with subsequent increases in protein synthesis, gluconeogenesis and/or ketogenesis. Amino acid can influence the rate of hepatic protein and RNA synthesis.

Publications:
Clin. Med. 77:26-29, 1970.
J. Infect. Dis. 122:159-169, 1970.
Amer. J. Clin. Nutr. 24:329-341, 1971.
Metabolism, 1971, In press.
Biochem. J., 1971, In press.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 — PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 01: Pathogenesis of Infection of Military Importance

Work Unit No. 096 01 009: Host Amino Acid, Protein and RNA Metabolism During Infectious Disease

Description:

Study changes in amino acids of blood and tissues in infectious disease or in conditions induced by other variables.

Progress, Part I:

Earlier data obtained by paper chromatographic separations of whole blood amino acids revealed that both vacterial and viral infections in man resulted in a drop in total blood amino acids[1-5] (J. Infect. Dis. 117. 346, 1967; Amer. J. Trop. Med. 16:769, 1967; New Eng. J. Med. 278:293, 1968; USAMU Annual Progress Rep. FY 1968, p. 36; USAMRIID Annual Progress Rep. FY 1969, p.37).

At 0730 hours fasting concentrations of 21 individual plasma amino acids were determined by ion exchange chromatography in daily serial samples from 8 volunteers infected with sandfly fever virus and compared to values obtained in 6 separate daily preexposure baseline measurements in each volunteer as well as to serial measurements in 3 unexposed control subjects (Medical Division Project No. FY 70-1 and 70-3). By 47 hr after inoculation and before the onset of fever or other clinical indications of infection, most individual plasma amino acids were significantly depressed below preexposure values[6] (USAMRIID Annual Progress Rep. FY 1970, p. 35). Such changes in plasma amino acids did not occur in control subjects. Reduction in amino acid concentrations persisted until after the lysis of fever and did not coincide in timing with alterations in white blood counts or serum Zn and Fe values. Since, urinary total nitrogen, urea and α-amino N were not altered during the course of sandfly fever in these subjects, the early decrease in plasma amino acid is not related to increased catabolism or excretion of amino acids. The observed depression in plasma amino acids began before any reduction in food intake due to anorexia. The magnitude of the amino acid depression during sandfly fever was far greater than that reported during starvation or protein deprivation in noninfected subjects[7,8] (J. Appl. Physiol. 25:52, 1968; Amer. J. Clin. Nutr. 23:986, 1970) and the sequence of changes in plasma valine, alanine, and glycine followed patterns different from those reported during starvation. It is postulated that unusually large quantities of certain plasma amino acids were taken up by the cells of key tissues during this infection and rapidly utilized for

protein synthesis, gluconeogenesis, and/or ketogenesis. Depression of those amino acids which are thought to be essential for protein synthesis[97] (Biochem. J. 111:703, 1969), especially the branch-chain group (valine, leucine and isoleucine) was somewhat greater than that observed for other amino acid groups.

Plasma phenylalanine responded in a manner different from that of the other amino acids; it was decreased on day 2 but by day 4 and 5 was significantly increased above preinfection values. This resulted in a significant increase in the phenylalanine-to-tyrosine ratio from 0.90 (range 0.62-1.17) in the preexposure period to 1.78 (range 1.37-2.64), during the febrile phase of sandfly fever. This measurement may be of value in detection of infectious illness.

In another study (Medical Division Project No. FY 70-8) the volunteers were given aspirin and propoxyphene HCl (Darvon^R) during the illness phase of the sandfly fever virus infection. Under these conditions, the clinical symptoms, as assessed by degree of febrile response and general malaise, were significantly reduced but the changes in plasma amino acids were similar to those observed in untreated volunteers exposed to the virus. Thus, the changes in plasma amino acid are not related to elevated body temperature.

Sequential changes in the concentration of individual serum free amino acids have, also, been determined in 11 volunteers who were exposed to 10^5 Salmonella typhosa organisms (a cooperative study with the Division of Infectious Diseases, University of Maryland School of Medicine, Contract No. DA49-193-MD-2867). On day 1 the concentration of total serum amino acid was significantly reduced below preexposure values. The levels returned to control values on day 3 but were again significantly depressed during the illness phase of the infection. On day 1 the branch-chain amino acids were significantly depressed but at all other time periods they, as well as the sum of the amino acid which are essential for protein synthesis, were at preexposure values. Serum alanine, glycine glutamine, proline and threonine were decreased at all time periods during the infection and did not return to normal concentrations until day 27. The marked depression of the serum glucogenic and ketogenic amino acids and the lesser affect on amino acids that are essential for protein synthesis were the opposite of the changes observed in volunteers infected with sandfly fever virus. It is postulated that during S. typhosa infection, gluconeogenesis and ketogenesis of amino acids are greater than during sandfly fever virus infection. It is also hypothesized that the flow of amino acids from muscle to liver is greater in volunteers infected with S. typhosa than with sandfly fever virus.

As was observed in the volunteers exposed to sandfly fever virus the phenylalanine-to-tyrosine ratio was significantly increased from 0.99 (range 0.64 - 1.18) during the control period to 2.04 (range 1.25 - 3.20) during the illness phase of typhoid fever. Thus, the changes in phenylalanine-tyrosine ratio were similar in both acute bacterial and viral infections.

Nine volunteers (Medical Division Project No. FY 71-2) exposed to $10^{6.4}$ media tissue culture infectious doses of adenovirus type 21 (ADV-21) excreted virus in stools and developed specific neutralizing antibodies. All of the volunteers remained afebrile and had no abnormalities in hematocrit, complete blood count, platelet count, total, direct and indirect bilirubin, serum glutamic oxaloacetic or pyruvic transaminases, alkaline phosphatase, blood urea nitrogen or urinalyses throughout the 28-day study. The plasma amino acids were depressed throughout the post-exposure period with maximal depressions on days 6 and 7. The greatest depression was observed in proline, 40% of preexposure concentrations in some volunteers. Plasma alanine, glutamine, valine and threonine were also depressed, especially during the later stages of the study. Plasma aspartate, tryptophan, phenylalanine, methionine and isoleucine were slightly elevated during the same period. The phenylalanine-to-tyrosine ratio was not significantly elevated in this study.

These data indicate that a mild asymptomatic viral infection can stimulate the movement of amino acids into certain cells with subsequent synthesis of proteins.

Summary, Part I:

Both viral and bacterial infections in man are characterized by an early depression in plasma free amino acids. The pattern of change in individual amino acids appears to be characteristic for the particular infection under study. The depression appears to result from a flow of these acids into cells of certain tissues. In acute bacterial and viral infections the plasma phenylalanine-to-tyrosine ratio is elevated during the height of illness.

Progress, Part II:

In order to understand the significance of the infection-induced depression of plasma amino acids, tissue levels were measured during infection in experimental animal models. The first model employed was subcutaneous injection of rats with *Diplococcus pneumoniae*. A statistical analysis of individual free amino acids of serum, liver and muscle was calculated from samples obtained at 4, 28 and 52 hr after exposure to 10^6 cells. Total free amino acids were significantly lower than control values at 28 and 52 hours. Those amino acids that are essential for protein synthesis[9/] were decreased at all 3 time periods tested. In liver, total free amino acids were decreased at 4 and 28 hr, but those acids involved in protein synthesis were increased at these time periods. In muscle, total free amino acids were markedly depressed at 4 and 28, with the greatest decreases being in glucogenic and ketogenic amino acids. From these observations, it is concluded that amino acids are being shunted from skeletal muscle to viscera where they are rapidly synthesized into proteins. To test this hypothesis, infected and noninfected

rats were injected with ^{14}C-1-aminocyclopentane (cycloleucine) (an amino acid analogue which is transported into cells but is not incorporated into protein) and with ^{3}H-leucine. At various times after exposure to D. pneumoniae, the rats were killed and tissue concentrations of labeled cycloleucine, and both free and protein bound leucine were measured. As the infection progressed, the concentration of hepatic cycloleucine increased while that in serum and muscle decreased. At 28 hr the livers from infected rats had 2 to 3 times as much cycloleucine as controls. The free ^{3}H-leucine content of liver, serum, and muscle was not significantly altered by infection. The amount of tritiated leucine bound to liver protein was slightly increased in infected animals; 3 times as much ^{3}H-leucine was incorporated into serum protein of infected rats and only half as much leucine was incorporated into muscle protein in the experimental group as compared to controls. These data support the conclusion that in infected animals amino acids are being shunted from muscle to tissues, such as liver, and are being rapidly synthesized into proteins. Since muscle protein synthesis is markedly reduced, a normal rate of catabolism would result in the breakdown of muscle protein and could supply the amino acids for rapid transport to liver.

Monkeys and rats were infected with Salmonella typhimurium, as the second model infection; sequential changes in individual serum amino acids were measured by ion exchange chromatography. The pattern of change observed in monkeys and rats was similar to that in volunteers exposed to S. typhosa. Rats infected with S. typhimurium and noninfected controls were injected with ^{14}C-cycloleucine. At 8 hr the liver of the infected rats had 3 to 4 times as much cycloleucine as controls, at 24 hr, 7-8 times. In rats infected with D. pneumoniae, an infection characterized by serum amino acid changes similar to those seen in volunteers exposed to sandfly fever virus, the maximum increase in cycloleucine content was only 2 to 3 times that of controls. These data support the hypothesis that during the course of an infection with S. typhosa the flow of amino acids from muscle to liver is much greater than in volunteers infected with sandfly fever virus.

In cooperation with the Animal Assessment Division we have developed a mask that will allow the measurement of the conversion of ^{14}C-alanine or pyruvate into ^{14}C labeled CO_2 and glucose. By these procedures we hope to assess the effect of an infection with D. pneumoniae and S. typhimurium on gluconeogenesis and ketogenesis.

Recently, it has been reported that a saline wash from incubated leukocytes will depress serum Zn[10,11/] (Fed. Proc. 29:297, 1970; Proc. Soc. Exp. Biol. Med. 133:128, 1970) and Fe[11,12/] (Proc. Soc. Exp. Biol. Med. 134: 1150, 1970) and will elicit a pyrogenic response in rabbits[13/] (Physiol. Rev. 40:580, 1960). The transport of ^{14}C-cycloleucine into liver cells of rats was significantly stimulated by injection of a crude extract of leukocytes; 1 hr postinoculation, the labeled cycloleucine content of liver was significantly elevated above control levels. Its concentration continued to increase at a logarithmic rate for the next 2 hr, at which time the

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cycloleucine content of the liver was twice that of controls. These data suggest that the leukocytic extract stimulates active transport of the amino acids and that the process follows first order kinetics. A heat inactivated (90C for 30 min) leukocytic extract or a homogenate of leukocytes did not stimulate the transport of cycloleucine into liver cells. Studies are currently in progress to isolate and characterize the mediator that will stimulate amino acid transport into liver cells.

Filtered serum from volunteers infected with S. typhosa was injected into recipient rats and stimulated the transport of cycloleucine into liver cells when compared to control sera similarly injected. Thus, the mediator was present in the serum of the infected volunteers. Procedures are currently being developed to allow for more sensitive detection of this mediator in the serum of infected individuals.

In contrast to changes in serum iron and zinc[14,15/] (Appl. Microbiol. 18:482, 1969; Proc. Soc. Exp. Biol. Med. 136:584, 1971), endotoxin of Escherichia coli or the double-stranded polynucleotides, poly I:C or poly A:U, did not stimulate the transport of cycloleucine into liver cells. These results suggest that the mediator for the stimulation of amino acids is different from the one that affects the depression of serum Zn and Fe.

Summary, Part II:

Evidence obtained from 2 model infections, D. pneumoniae and S. typhimurium in rats, support the hypothesis that infection-related changes in plasma amino acids reflect a flow of amino acids from muscle to tissues, such as liver, and their rapid utilization for protein synthesis, gluconeogenesis, and/or ketogenesis. This flow of amino acids is stimulated by a mediator that is released from leukocytes. Sera from volunteers exposed to S. typhosa contained a similar mediator for amino acid transport.

Progress, Part III:

Recently, it has been demonstrated that amino acids can stimulate formation of hepatic polyribosomes and the rate of in vitro protein synthesis[16,17,18/] (J. Molec. Biol. 34:199, 1968; J. Biol. Chem. 243:1123, 1968; Proc. Soc. Exp. Biol. Med. 135:180, 1970). When weanling rats were fed a low protein diet for 28 days, the concentration of hepatic free amino acid was significantly reduced[19/] (Biochem. J. 107:615, 1968). A similar model was utilized to determine whether the reduced hepatic amino acid content would have an in vivo effect on protein and RNA synthesis.

Weanling (23-day old) rats were fed either an amino acid-deficient diet (6% casein) or a diet containing an adequate amount of protein (18% casein) for 28 days. Hepatic cells from animals fed the deficient diet were characterized by markedly lower concentrations of protein and RNA in all

cellular fractions as compared with cells from control rats. The bound ribosomal RNA fraction was decreased to the greatest degree, while the free ribosomal concentrations were only slightly less than in control animals. A good correlation was observed between the in vivo rate of hepatic protein synthesis and the cellular protein content of the liver. Both in vivo and in vitro rates of protein synthesis were directly correlated with the hepatic concentration of individual free amino acids which are essential for protein synthesis.[9/] The reduced protein synthetic ability of the ribosomes from the liver of protein-deprived rats was related to a decrease in the number of active ribosomes and heavy polyribosomes. The lower ribosomal content of the hepatocytes was correlated with the reduced concentration of essential free amino acids. In the protein-deprived rats, the rate of accumulation of newly synthesized cytoplasmic r-RNA was markedly decreased as compared to control animals. From these data it was concluded that amino acids regulate protein synthesis (1) by affecting the number of ribosomes that actively synthesize protein and (2) by inhibiting the rate of synthesis of new ribosomes. Both of these processes may involve the synthesis of proteins which have a rapid rate of turnover.

Studies were initiated in rats infected with D. pneumoniae, to determine whether the flow of amino acids into liver had similar effects on protein and RNA metabolism. Preliminary observations indicate that the percentage of hepatic polyribosomes was decreased 4 hr postexposure but had returned to control period concentrations at 28 and 52 hr. Pair-fed controls had gradual decreases in percentages of hepatic polyribosomes. At 28 and 52 hr in vitro protein synthetic ability of ribosomes, the number of active ribosomes, and the concentrations of ribosomes in liver were all increased above control values. These data suggest that the increased flow of amino acids into liver stimulated protein and RNA synthesis.

Summary, Part III:

Amino acids have a marked in vivo regulatory effect on both hepatic protein and ribosome synthesis. A similar mechanism appears to explain the infection related stimulation of hepatic and serum proteins.

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2. Burghen, G. A., W. R. Beisel, P. J. Bartelloni. 1970. Influences of chloramphenicol administration on whole-blood amino acids in man. Clin. Med. 77:26-29.

3. Feigin, R. D., W. R. Beisel and R. W. Wannemacher, Jr. 1971. Rhythmicity of plasma amino acids and relation to dietary intake. Amer. J. Clin. Nutr. 24:329-341.

4. Wannemacher, Jr., R. W., R. S. Pekarek, P. J. Bartelloni, R. T. Vollmer, and W. R. Beisel. 1971. Changes in individual plasma amino acids following experimentally-induced sandfly fever virus infection. Metabolism. In press.

5. Wannemacher, Jr., R. W., C. F. Wannemacher, and M. B. Yatvin. 1971. Amino acid regulation of RNA and protein synthesis in the liver of rats. Biochem. J. In press.

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2. Feigin, R. D., R. F. Jaeger, R. W. McKinney, and A. C. Alevizatos. 1967. Live, attenuated Venezuelan equine encephalomyelitis virus vaccine, II. Whole-blood amino acids and fluorescent-antibody studies following immunization. Amer. J. Trop. Med. 16:769-777.

3. Feigin, R. D., A. S. Klainer, W. R. Beisel and R. B. Hornick. 1968. Whole-blood amino acids in experimentally induced typhoid fever in man. New Eng. J. Med. 278:293-298.

4. U. S. Army Medical Unit. 1 July 1968. Annual Progress Report, FY 1968. p. 39 to 44, Fort Detrick, Maryland.

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ANNUAL PROGRESS REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 01: Pathogenesis of Infection of Military Importance

Work Unit No. 096 01 010: Effect of Ionizing Radiation on Infection and Immunity

Reporting Installation: U. S. Army Medical Research Institute of Infectious Diseases
Fort Detrick, Maryland

Divisions: Animal Assessment and Medical

Period Covered by Report: 1 July 1970 to 30 June 1971

Professional Authors: Richard O. Spertzel, Lt Colonel, VC (I)
John C. Holder, Major, MC (II)
Douglas W. Mason, Captain, VC (I)
James W. Brown (I, II)

Reports Control Symbol: RCS-MEDDH-288(R1)

Security Classification: UNCLASSIFIED

42

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION: DA OL0883
2. DATE OF SUMMARY: 71 07 01
REPORT CONTROL SYMBOL: DD DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 01 06	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	01	010
b. CONTRIBUTING				
c. ~~//////~~	CDOG 1212b(9)			

11. TITLE (Precede with Security Classification Code): (U) Effect of Irradiation on Infection and Immunity for EW and RW Defensive Research (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 014100 Radiobiology; 003500 Clinical Medicine; 004900 Defense

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
67 07	CONT	DA	C. In-house

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER: NA
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	2	30
	CURRENT 72	2	31

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, Md 21701

RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

21. GENERAL USE

20. PERFORMING ORGANIZATION

NAME: Animal Assessment and Medical Div USAMRIID
ADDRESS: Fort Detrick, Md 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Spertzel, R. O.
TELEPHONE: 301 663-4111 Ext 7244
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Holder, J. C.
NAME: Mason, D. W. DA

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Radiation; (U) Mice; (U) Infectious diseases; (U) Vaccine; (U) Encephalomyelitis, equine (VEE); (U) Military Medicine

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Investigate interrelationships between acute or chronic irradiation and disease processes.

24 (U) Acute or protracted whole body irradiation is delivered to selected animal species before, simultaneously with, or after infection. Clinical and immune responses are observed and measured serially.

25 (U) 70 07 - 71 06 - I. Studies have been conducted to determine the effects of sublethal, acute, total-body irradiation (600 r) on the immune response of mice to an attenuated strain of Venezuelan equine encephalomyelitis (VEE). The data suggest that irradiation before or after inoculation with attenuated VEE delays the onset of protection against virulent challenge. The shorter the time interval between irradiation and vaccination, the longer the delay of onset of protection. Irradiation also markedly depressed the HI antibody response to attenuated VEE, the greatest depression occurring with shortest time for irradiation to vaccination.

When the attenuated VEE was inoculated into previously irradiated mice, sporadic, but significant, deaths occurred, but did not seem to be dose-dependent on the VEE.

Irradiation did not alter susceptibility of mice to the virulent challenge.

II. Modification of equipment for chronic irradiation is being modified since dosages from the existing 60-Cobalt Source were variable.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 01: Pathogenesis of Infection of Military Importance

Work Unit No. 096 01 010: Effect of Ionizing Radiation on Infection and Immunity

Description:

Investigate interrelationships between acute and chronic irradiation and disease processes.

Progress, Part I:

Several investigators have observed altered host resistance to infection following irradiation. Many have shown that animal host systems were more susceptible to infection when they were irradiated[1,2] (Probl. Med. Virol. 5:138, 1953; Med. Radiol. 7(7):62, 1962). Other investigators have shown the opposite to be true; that is, irradiation of the host animal has seemed to increase the animal's resistance to virulent organisms[3-6] (Med. Radiol. 5(8):82, 1960; J. Immun. 87:682, 1961; Proc. Soc. Exp. Biol. Med. 108:183, 1961; J. Hyg. 66:355, 1968). Similarly, irradiation has been reported to enhance antibody response[7] (J. Immun. 91:761, 1963), depress the antibody response[8] (Radiat. Res. 20:383, 1963), or merely delay antibody response[9] (Contract 3A-014501 Final Report, 1 Sep 1969), which varies with time of irradiation,[8] dose of irradiation[9] or state of the antigen[10] (Radiat. Res. 36:98, 1968). Thus, in a situation where personnel have been exposed to acute irradiation, we must know: when will a vaccine be effective, and is a live attenuated or a killed vaccine preferable? To answer some of these questions relative to protective immunization, a program has been established to study the interrelationships of vaccine administration, dose of irradiation and time-dose relationship of vaccine and irradiation to immunity.

White Swiss mice were irradiated at exactly 30 days of age and inoculated with live, attenuated Venezuelan equine encephalomyelitis (VEE) vaccine strain virus (National Drug Co. Lot 4 or 5) at various days postirradiation. Irradiation was accomplished as a single whole-body exposure, employing a 1-MEV unit operated at 3 MA, at a distance of approximately 117 cm, and a dose rate with inherent filtration of 50 r/min (air dose). The mice were irradiated in a plexiglas chamber; each mouse was placed in an individual compartment to inhibit radiation avoidance. Following irradiation, the mice were given water containing 5 ppm Cl. Water and water bottles were changed daily. Irradiation dose-response curves of mice were obtained (Table I) and the mean lethal dose ($LD_{50(30)}$) that will kill mice within 30 days was determined to be 670 r by probit analysis. Thus, in these initial studies, a dose of 600 r irradiation was selected. Lower doses of irradiation will be studied later. To eliminate some of the variation between experiments, the days of irradiation were always varied, but all vaccinations and challenges for each particular experiment were accomplished on the same day.

TABLE 11. EFFECT OF TIMING [a/] OF 600r IRRADIATION AND TRINIDAD VEE CHALLENGE ON VEE-IMMUNIZED MICE

DAY OF VEE CHALLENGE POSTVACCINATION	SURVIVORS/TOTAL BY DAY OF IRRADIATION PREVACCINATION												
	-21	-14	-12	-10	-8	-7	-4	-3	-2	-1	0	+1	Vaccine Control
+ 1		0/5	0/5	0/9	0/6					0/7	0/7		[illegible]
+ 2		1/7	0/6	0/9	0/7					0/7	0/7		0/[illegible]
+ 3		0/9	0/6	0/10	0/9					0/7	0/7		0/5
+ 4		0/8	0/7	0/3	0/10					0/7	0/7		2/[illegible]
+ 5	22/24	16/26	3/20	0/17	0/14	0/5	0/2	0/11	0/22	1/30	0/20	0/8	35/42
+ 6		5/8	4/6	0/6	0/9					0/7	0/7		4/[illegible]
+ 7	20/23	26/34	11/20	13/28	9/15	5/14	0/3	1/16	1/26	0/37	2/42	0/12	50/53
+10	12/12	10/11				0/2	0/3	0/3	1/13	0/2	2/6		21/22
+14	20/21	23/24	7/8	13/18	9/10	24/32	18/22	19/30	15/18	14/23	11/14	7/11	33/35
+21		10/10	9/11	14/18		26/32		10/10	9/10	12/12	7/8		12/12
+28		9/9		4/4		11/12		11/12	11/11	8/11	9/10		12/12

% Survivors

+ 1		0	0	0	0					0	0		0
+ 2		14	0	0	0					0	0		0
+ 3		0	0	0	0					0	0		0
+ 4		0	0	0	0					0	0		33
+ 5	91	61	15	0	0	0	0	0	0	3	0	0	83
+ 6		62	66	0	0					0	0		66
+ 7	86	76	55	46	60	36	0	6	3	0	47	0	94
+10	100	90				0	0	0	7	0	33		95
+14	96	95	87	72	90	75	81	63	83	60	78	63	94
+21		100	81	77		81		100	90	100	87		100
+28		100		100		91		91	100	72	90		100

a. Negative numbers indicate irradiation before vaccination, positive numbers indicate irradiation following vaccination. Day 0 is day of immunization.

TABLE I. IRRADIATION DOSE RESPONSE TABLE OF 30-DAY-OLD MICE

AIR DOSE IN ROENTGENS	DEAD/TOTAL	$LD_{50(30)}$
500	0/24	
550	2/24	
600	4/24	
650	4/24	
700	15/24	670
750	23/24	
800	24/24	
Controls	0/24	

Previous studies[11/] (USAMU Annual Progress Report, FY 1965, p. 143) showed that whole-body irradiation of mice at 500 r delivered 8-48 hr prior to immunization with a sufficiently large dose of living attenuated VEE vaccine, had no effect on resistance to challenge 5-14 days later with the parent Trinidad strain VEE virus.

In order for us to clarify and broaden previous data, the first set of experiments was designed so that groups of mice were irradiated at intervals of time that ranged from 21 days before vaccination to 1 day after vaccination with 5×10^3 mean guinea pig intraperitoneal immunizing dose ($GPIPID_{50}$) of attenuated VEE. The immunized mice were challenged with 10^3 mean mouse intracranial (IC) lethal dose ($MICLD_{50}$) of Trinidad strain VEE from 1-28 days post-vaccination. The results are summarized in Table II.

From these results, it appears that 600 r of irradiation delay the onset of protection in mice, followed by a general trend to return to control levels. It also appears that the shorter the time interval between irradiation and vaccination, the longer the onset of protection is delayed.

Since it was apparent that immunity was delayed when irradiation preceded vaccination, the alternate experiment of irradiation following vaccination was performed. The results are summarized in Table III.

TABLE III. EFFECT OF POSTVACCINATION IRRADIATION ON THE IMMUNITY PRODUCED BY ATTENUATED VEE IN MICE AGAINST TRINIDAD VEE CHALLENGE

DAY OF VEE CHALLENGE POSTVACCINATION	SURVIVORS/TOTAL BY DAY OF IRRADIATION POSTVACCINATION					
	0	+1	+2	+3	+4	Vaccine Control
+ 5	4/11	1/12	3/12	5/12	10/12	11/12
+ 7	5/8	9/12	7/12	3/11	7/12	12/12
+14	5/9	3/7	3/7	4/7	4/7	13/14

It can be seen that irradiation following vaccination also depresses the immune response produced by this attenuated virus.

An ancillary observation made throughout the experiments was that the attenuated form of VEE causes some deaths in irradiated mice. To pursue this observation, experiments were designed in which irradiated mice were inoculated with 50 and 5000 $GPIPID_{50}$ of live VEE vaccine and then observed for 14 days for deaths. Pooled results are summarized in Table IV.

To determine if the mortality induced by the attenuated form of VEE was dose-dependent, an experiment was designed in which attenuated VEE at concentrations from 10^1 to 10^6 $GPIPID_{50}$ were inoculated into irradiated mice. Table V presents the results.

It appears from the data that TC-83-VEE-induced mortality in irradiated mice is a sporadic, but significant, occurrence; it is not TC-83-dose-dependent.

It is unknown what the role of interferon is in the resistance produced by the VEE vaccine strain. Virology Division has found resistance in guinea pigs and hamsters as early as 8 hr after immunization against an intraperitoneal (IP) challenge with Trinidad VEE. Our data suggest that protection against an IP challenge of Trinidad VEE can be seen as early as 2 days following administration of the vaccine (Table VI).

Previous studies[11] have shown that 500 r of irradiation definitely inhibited resistance to Trinidad VEE at 1 and 3 days postimmunization. It is unknown what effect irradiation has on VEE-vaccine-strain-induced interferon. To evaluate this, a project was designed in which mice were irradiated 0-14 days before vaccination with 5×10^3 $GPIPID_{50}$. These mice were then bled out 1-5 days postvaccination; the sera were sent to Virology Division for interferon assay; results are not yet available. Additional groups of mice, simi-

TABLE IV. VEE VACCINE STRAIN INDUCED MORTALITY OF IRRADIATED MICE.

CHALLENGE DOSE ($GPIPID_{50}$)	VACCINE[a] CONTROLS	DAYS OF IRRADIATION PREVACCINATION								
		-8	-7	-6	-5	-4	-3	-2	-1	0
						Survivors/Total				
50	12/12	18/23	49/81	50/67	37/48	44/70	35/60	15/24	11/12	9/12
5000	12/12	20/23	55/82	43/65	29/47	45/60	32/60	13/24	6/12	12/12
0[b]		24/24	67/77	41/51	35/48	48/54	31/53	7/12	4/6	5/6
						% Survival				
50	100	78	60	74	77	62	58	62	91	75
5000	100	86	67	66	61	75	53	54	50	100
0		100	87	80	72	88	58	58	66	83

a. Vaccine Controls - unirradiated, vaccinated mice.

b. Radiation Controls = irradiated mice.

larly irradiated, will be evaluated with respect to survival to virulent challenge, so that interferon levels and protection can be correlated.

TABLE V. DOSE-RESPONSE CURVE OF VEE VACCINE-INDUCED MORTALITY IN IRRADIATED MICE

VEE VACCINE DOSE ($GPIPID_{50}$)	DAY OF IRRADIATION PREVACCINATION -10	-7	-6	-5	-4	-3	-1	VACCINE CONTROLS
	Survivors/Total							
10^6	7/9	11/12	11/12	10/11	11/12	11/12	10/12	12/12
10^5	7/11	12/12	10/12	9/12	11/12	8/12	5/12	11/12
10^3	10/12	9/11	8/12	8/12	9/12	7/12	6/12	12/12
10^1	11/12	8/12	10/11	11/11	8/12	7/12	10/12	12/12
0	8/12	8/12	9/9	9/11	9/12	11/12	9/12	
	% Survival							
10^6	77	92	92	91	92	92	83	100
10^5	64	100	83	75	92	67	42	92
10^3	83	82	67	67	75	58	50	100
10^1	92	67	91	100	67	58	83	100
0	67	67	100	82	75	92	75	

The effect of irradiation on hemagglutination-inhibition (HI) antibody titers produced by the vaccine strain in mice had been previously done in this Institute in 1963[12] (USAMU Annual Progress Report, FY 1963, p. 233) and 1966[13] (USAMU Annual Progress Report, FY 1966, p. 147). On both occasions, the irradiation preceded vaccination by 1 day or less. They found that the HI titers of pooled mouse sera were lower in irradiated animals than in non-irradiated controls. The time of inoculation of the virus as related to irradiation did not influence the HI response significantly, in contrast to the quantity of virus inoculated in their experiments. To expand on these results, a preliminary experiment was designed where mice were irradiated with 600 r 0-14 days before administration of 5 x 10^3 $GPIPID_{50}$ of VEE vaccine strain virus. Groups of mice were then bled out on days 6-14 postvaccination (Table VII).

TABLE VI. COMPARISON OF EARLY VEE-INDUCED RESISTANCE AGAINST AN IP AND IC CHALLENGE OF MICE WITH TRINIDAD VIRUS

CHALLENGE ROUTE	SURVIVORS/6 MICE BY DAY OF CHALLENGE POSTVACCINATION					
	1	2	3	4	5	6
Vaccinated						
IC	0/4	0	0/5	2	0	5/5
IP	0	3	6	5	5	5
Control						
IC	0/5	0	0	1	-	0
IP	0	1/5	0	0	-	0

TABLE VII. EFFECT OF PRIOR IRRADIATION ON VEE HI ANTIBODY TITERS

DAY OF IRRADIATION BEFORE ADMINISTRATION OF TC-83 VEE	RECIPROCAL MEAN HI TITER BY DAY OF BLEEDING POSTVACCINATION								
	+6	+7	+8	+9	+10	+11	+12	+13	+14
-14	160	35	57	204	243	279	279	279	160
-12	28	90	320	113	320	320	160	139	279
-10	25	105	121	106	211	171	211	320	260
- 8	25	20	36	130	98	171	184	121	130
- 1	25	20	21	368[a]	24	43	25	70	65
0	24	11	20	135[a]	28	28	26	21	33
Vaccine Controls	1810	3880	2390	2150	905	1280	1190	1940	1080

a. These seem to be aberrant figures.

The data show that irradiation very markedly decreases HI titers in mice. As expected, in contrast to the previous work, the shorter the time interval between irradiation and inoculation of TC-83, the lower the titer observed. Further work will be done in this area to attempt to correlate HI titer

studies with serum neutralization and survivor studies.

Previous studies[14] (USAMU Annual Progress Report, FY 1964, p. 17) showed that when mice were irradiated and then challenged with Trinidad strain VEE, the mice became slightly more resistant to the challenge, as shown by median dose response. A similar project was designed in which mice were irradiated at varying times between 2 and 14 days before being challenged with serial 10-fold dilutions of Trinidad strain VEE.

In contrast to the previous data, we showed a general trend, albeit not a statistically significant effect, of increased susceptibility as the irradiation-to-challenge time interval decreased.

An $LD_{50(30)}$ study was done on 30-day-old Swiss Webster mice, obtained from the Fort Detrick Animal Farm Division, to determine if they were suitable for acute radiation studies (Table VIII).

TABLE VIII. DOSE-RESPONSE TABLE OF FORT DETRICK 30-DAY-OLD MICE

AIR DOSE (ROENTGENS)	DEAD/TOTAL	$LD_{50(30)}$
500	6/48	
550	16/48	
600	30/48	
650	28/48	615 r
700	40/48	
750	44/48	
Room Controls	0/8	

By probit analysis, the $LD_{50(30)}$ was determined to be 615 r for the Fort Detrick mice. Because the mean $LD_{50(30)}$ is approximately 55 r lower than on the mice currently being used, we are unable to utilize the Fort Detrick mice.

Similarly, because of an abundance of adult (2-9-mon-old) CD-1 mice, an $LD_{50(30)}$ study was carried out with them (Table IX).

As expected, the $LD_{50(30)}$ for the adult mice was approximately 760 r, about 85 r higher than for the 30-day-old mice. The adult mice will be used in future experiments, but the irradiation dose will be increased to 650 r.

TABLE IX. DOSE-RESPONSE TABLE OF ADULT MICE

AIR DOSE (ROENTGENS)	DEAD/TOTAL	$LD_{50(30)}$
650	3/24	
700	6/24	
750	9/24	760 r
800	23/24	
850	24/24	
Controls	0/8	

Summary, Part I:

Studies have been conducted to determine the effects of sublethal, acute, total-body irradiation on the immune response of mice to an attenuated strain of VEE virus. Our work indicates that irradiation before or after vaccination delays the onset of protection from the parent Trinidad strain VEE. The shorter the time interval between irradiation and vaccination, the longer the delay of onset of protection.

Irradiation markedly depresses the HI response in mice immunized with live, attenuated VEE vaccine. As observed in the survivor studies, the shorter the time interval between irradiation and vaccination, the greater the depression of the HI titers.

It has also been noted that when the attenuated form of VEE is inoculated into irradiated mice, sporadic, but significant numbers of, deaths occur. These deaths do not appear to be dependent on the VEE dose administered.

Irradiation seems to have no effect on a mouse's susceptibility to the virulent form of VEE, as indicated by the median lethal dose.

Progress and Summary, Part II:

In chronic radiation studies, no work is being conducted pending approval and completion of modifications to the ^{60}Co irradiator.

Publications:

None.

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ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 01:	Pathogenesis of Infection of Military Importance
Work Unit No. 096 01 011:	Rapid Electron Microscopic Assay for Virus Particles
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Division:	Physical Sciences
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Author:	Anne Buzzell, Ph.D.
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY		1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
		DA OL0810	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 01 06	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	01	011
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	CDOG 1212b(9)			

11. TITLE (Precede with Security Classification Code)* (U) Rapid Electron Microscopic Assay for Virus Particles of Diseases of Military Medical Importance (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*
003200 Biochemistry; 003500 Clinical Medicine; 004900 Defense

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
62 02	CONT	DA	C. In-house

17. CONTRACT GRANT
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER*:
c. TYPE: NA
d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	1	15
	CURRENT 72	1	36

19. RESPONSIBLE DOD ORGANIZATION
NAME:* USA Medical Research Institute of Infectious Diseases
ADDRESS:* Fort Detrick, Md 21701
RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
NAME:* Physical Sciences Division USAMRIID
ADDRESS:* Fort Detrick, Md 21701
PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME:* Buzzell, A.
TELEPHONE: 301 663-4111 Ext 6237
SOCIAL SECURITY ACCOUNT NUMBER:
ASSOCIATE INVESTIGATORS
NAME:
NAME: DA

21. GENERAL USE
Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Virus; (U) Electron microscopy; (U) Negative staining; (U) Military Medicine

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) To develop a rapid electron microscopic assay for virus particles.

24 (U) A method is being developed which involves the transfer of virus from a millipore filter to an electron microscope grid with negative staining.

25 (U) 70 07 - 71 06 - Pending completion of a series of papers on the model for mechanism of membrane transport, experimental work on the electron microscopic assay for virus particles has been temporarily halted. As predicted earlier, much of the literature survey conducted in connection with the model for transport across biological membranes has provided numerous ideas concerning the probable relative importance of, and means of dealing with, various factors which have been found to affect the ability of a salt solution, particularly one with a large, highly charged anion such as phosphotungstate, to pass through a small-pored membrane such as a millipore filter.

*Available to contractors upon originator's approval

DD FORM 1498, 1 MAR 68 — PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 01: Pathogenesis of Infection of Military Importance

Work Unit No. 096 01 011: Rapid Electron Microscopic Assay for Virus Particles

Description:

To develop a rapid electron microscopic assay for virus particles.

Progress and Summary:

Experimental work on the electron microscopic assay for virus particles has been temporarily halted, pending completion of a series of papers on the model for the mechanism of membrane transport. As predicted earlier, much of the literature survey conducted in connection with the model for transport across biological membranes has provided numerous ideas concerning the probable relative importance of, and means of dealing with, various factors which have been found to affect the ability of a salt solution, particularly one with a large, highly charged anion such as phosphotungstate, to pass through a small pored membrane such as a millipore filter. Transfer of the virus from some sort of small-pored, rigid filter by flow through the filter of the "negative stain" solution is an essential step in making the virus assay suitable for diagnosis. As pointed out in earlier reports it should be feasible to concentrate the virus enough, by funneling it down into the filter through a tiny hole in a conical centrifuge cell, to detect virus at a concentration as low as 10^2 particles/ml using, 1 ml of infected serum.

Publications:

None

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ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 01:	Pathogenesis of Infection of Military Importance
Work Unit No. 096 01 012:	Biophysical Study of Membrane Transport in Infection
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Division:	Physical Sciences
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Authors:	Anne Buzzell, Ph.D.
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
	DA OL0889	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 01 06	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	01	012
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1212b(9)			

11. TITLE (Precede with Security Classification Code) (U) Biophysical Studies of Membrane Transport in Infections of Military Medical Importance (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
002300 Biochemistry; 010100 Microbiology; 003500 Clinical Medicine; 004900 Defense

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
61 07	CONT	DA	C. In-house

17. CONTRACT GRANT
a. DATES/EFFECTIVE EXPIRATION
b. NUMBER
c. TYPE NA d. AMOUNT
e. KIND OF AWARD f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	1	30
	CURRENT 72	1	36

19. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, Md 21701

RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
NAME: Physical Sciences Division USAMRIID
ADDRESS: Fort Detrick, Md 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Buzzell, A.
TELEPHONE: 301 663-4111 Ext 6237
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME:
NAME: DA

21. GENERAL USE
Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Latex particles; (U) Membrane; (U) Pore; (U) Phospholipid; (U) Ion; (U) Chelate; (U) Ultracentrifugation; (U) Micelle; (U) Military Medicine

23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Characterize various toxins or other inert molecules which have biologic activity.

24 (U) Ultracentrifugation will be used to study the interactions of toxins and other inert molecules with the host tissues.

25. (U) 70 07 - 71 06 - After completing the paper, now in preparation, which will describe the basic membrane model, and demonstrate the ability of the model to account for a wide variety of transport phenomena associated with cell membranes described in the literature, new applications of the model will be sought in areas related to pathogenesis. A preliminary survey suggests a wealth of applications will be found.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 01: Pathogenesis of Infection of Military Importance

Work Unit No. 096 01 012: Biophysical Study of Membrane Transport in Infection

Description:

Characterize various toxins or other inert molecules which have biological activity.

Progress:

Work has continued on the theoretical model of the biological membrane[1] (USAMRIID Annual Progress Rep. FY 1970, p. 49) in which the mechanism for cation transport is a soap micelle enclosed by linear micelles of phosphatidic acid joined by Ca ions, with ATPase nearby to control transport of Na or K ions by compressing the soap. Accordingly, the enzyme, which is tightly bound in the membrane phospholipid, would not have to shuttle across the membrane as in the carrier protein theory[2] (Ann. Rev. Physiol. 32:21, 1970). Basic and long-chain neutral amino acids, which share a common route of entry[3] (Science 151:1010, 1966), should also be able to pass through the soap. An extension of the cation transport model, described elsewhere[4] (CES Annual Rep. FY 1970, p. 75) can also account for the final stages of assembly of myxovirus particles visible by electron microscopy.

The model has now been developed to include transport systems for other materials based on the structure of the phospholipid portion of the membrane. The basic idea for lecithin membranes, as described earlier,[1] was a linear micelle made up of dimers linked by hydrogen bonding between the phosphate residues, the dimers being spaced by the kinks in unsaturated chains of the fatty acid residues. The micelles, being fringed by amines, could serve to anchor the negatively charged "zippered" soap micelle. Since cholesterol and phospholipids combine in definite proportions to form stable membranes in vitro and since cholesterol is hydrophobic, bulky and uncharged, the molecule seemed likely to function in vivo as a spacer between highly charged phospholipid micelles. Furthermore, observations in vitro indicate that cholesterol forms a definite complex[5] (Biochim. Biophys. Acta 135:11, 1967), possibly using its lone hydroxyl group to form a hydrogen bond with the charged phosphate residues of the phospholipid dimers. Hydrogen bonding to the neutral phosphate residue being weaker, pores would be left in the membrane, having in the case of lecithin a rim of positive charges to attract anions to the entry site. Specificity of anion transport can be accounted for by well known differences between the properties of quaternary and primary amines on phosphatidyl ethanolamine.

With the recent acquisition of atomic models the proposed arrangement for the linear micelles could be tested directly. The optimum arrangement found for lecithin dimers with fatty acid chains in the stable "zig-zag" configuration do have kinked chains directed properly for linear micelle formation, when unsaturated fatty acids are in the central position, as is the case for lecithin. It is noteworthy that proper direction of the kink also requires the odd number of CH_2 residues between the carboxyl and the unsaturated bond which is characteristic of the fatty acids in phospholipids. Also, the dimer configuration leaves exposed only the ester bond of the central fatty acid, in accord with the susceptibility of this fatty acid to phospholipase action[6/] (Ann. Rev. Biochem. 35:157, 1966). A model of cholesterol, hydrogen bonded to the free phosphate oxygen, can be oriented so that it fits snugly in the niche formed by the fatty acid chains at the side of the dimer, the lone unsaturated bond of cholesterol being needed for best fit. Lecithin pores constructed with oleic acid spacing the dimers and cholesterol spacing the micelles proved big enough to admit Cl ions, but not Br, as would be required for the membranes of many cells.

In dimers of phosphatidyl serine the serine residues can be rotated to exchange positions of the amine and carboxyl. However, with dimers spaced by oleic acid, hydrogen bonding is possible between carboxyls and amines of neighboring dimers in the micelle. With this arrangement the positions of the remaining amines and carboxyls are fixed so as to permit attachment of amino acids, oriented over the pore and ready for entry when loosened by some motion of the membrane. Furthermore, steric hindrance would limit this route to L-isomers with short chains. If serine residues are rotated to bring both carboxyls between the dimers, chelation of divalent cations can occur, with a specificity and pH dependence in accord with known data.

The orientation of hydroxyls in inositol pairs, with ring planes parallel, attached to hydrogen bonded phosphatidyl inositol (PI) dimers can account in every detail for the specificity of sugar transport in the gut[7/] (Physiol. Rev. 40:789, 1961), if dimers of neighboring micelles are located opposite one another, an arrangement to be expected if glycoprotein molecules are inserted between the ends of the micelles with side chains attached to the terminal inositol pairs[4/]. Glycoproteins may also be needed as spacers at the edge of PI regions, since PI micelles cannot mesh charges with those of other phospholipids, the PI dimers being spaced far apart by the highly kinked chains of arachadonic acid, a spacing needed to prevent misattachment of the sugars. Attachment of glycoprotein to the inositol pairs changes their orientation, which would alter the specificity of sugar transport making it similar to that observed in red blood cells[8/] (Pharmacol. Rev. 13:39, 1961), which have relatively little PI.

Cholesterol binding to phospholipids containing arachadonic acid is weak,[5] so the gap between PI micelles, being empty, could be regulated by nearby soap micelles[4] through cation binding.[1] Actively transported sugars require Na, and are blocked by K, suggesting that size of a sugar determines whether it will accumulate against a concentration gradient in gut cells, where K concentration is high; transport specificity does indeed show that these sugars must attach with the long axis across the gap. In membranes with PI micelles too far apart, insulin might increase sugar transport by penetrating the soap. The polypeptide would of course be ineffective in gut cells where entry of amino acids inhibits sugar transport.[3]

Summary:

The basic features of the theoretical model of the biological membrane required to account for transport of all types of ions and of cell metabolites are now complete. Transport of monovalent cations (and exclusion of divalent) can be accounted for by a soap micelle enclosed by linear micelles of phosphatidic acid which are closed in zipper-fashion by Ca ions. Transport of long chain neutral and basic amino acids can also be explained by this mechanism and a number of polypeptide hormones insulin in particular may affect the cell at this site. Transport of other materials can be accounted for quantitatively by the sort of pores to be expected in a membrane built up of linear micelles of the other phospholipids with cholesterol acting as a spacer. Molecular models have been built of such micelles for all the principal phospholipids and the pores constructed with them do have the properties required to account quantitatively for the transport of anions, small neutral and acidic amino acids and sugars.

Publications:

None

LITERATURE CITED

1. U. S. Army Medical Research Institute of Infectious Diseases. 1 July 197 . Annual Progress Report, FY 1970. p. 49 to 53. Fort Detrick, Maryland.

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8. LeFevre, P. G. 1961. Sugar transport in the red blood cell; Structure-activity relationships in substrates and antagonists. Pharmacol. Rev. 13:39-70.

ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 01:	Pathogenesis of Infection of Military Importance
Work Unit No. 096 01 013:	Host Lipids in Infectious and Toxic Illness
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Division:	Physical Sciences and Animal Assessment
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Authors:	Robert H. Fiser, Jr., Major, MC Joseph C. Denniston, Captain, VC Robin T. Vollmer, Captain, MC William R. Beisel, M.D.
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

66

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
	DA OL0900	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 01 06	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	01	013
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	CDOG 1212b(9)			

11. TITLE (Precede with Security Classification Code)*
(U) Host Lipids in Infectious and Toxic Illnesses for Defense Against BW (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*
003500 Clinical Medicine; 002300 Biochemistry; 004900 Defense

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
69 07	CONT	DA	C. In-house

17. CONTRACT GRANT
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER*
c. TYPE: NA d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	2	60
	CURRENT 72	2	85

19. RESPONSIBLE DOD ORGANIZATION
NAME:* USA Medical Research Institute of Infectious Diseases
ADDRESS:* Fort Detrick, Md 21701
RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
NAME:* Physical Sciences and Animal Assessment Divisions
ADDRESS:* USAMRIID
Fort Detrick, Md 21701
PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME:* Fiser, Jr., R. H.
TELEPHONE: 301 663-4111 Ext 5158
SOCIAL SECURITY ACCOUNT NUMBER:
ASSOCIATE INVESTIGATORS
NAME: Denniston, Jr., J. C.
NAME: Beisel, W. R. DA

21. GENERAL USE
Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Lipids; (U) Lipoproteins; (U) Free fatty acid; (U) Cholesterol; (U) Triglycerides; (U) Endotoxin; (U) Bacterial illness; (U) Viral illness; (U) Military Medicine

23. TECHNICAL OBJECTIVE.* 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Study early changes in lipid metabolism during infectious and toxic illness.

24 (U) Measure serum lipids and lipoproteins during induced illness and examine the kinetics of fatty acid metabolism.

25 (U) 70 07 - 71 06 - In volunteers infected with sandfly fever virus, altered patterns of lipid transport preceded the onset of clinical illness. Marked effects of both carbohydrate and lipid fuel stores were seen; these varied with the stage of the illness. The data are consistent with an increased utilization and dependence on body fat stores for energy during this mild viral illness.

Monkeys infected with either Diplococcus pneumoniae or Salmonella typhimurium or intoxicated with endotoxin derived from S. typhimurium exhibited an increased turnover of free fatty acids, triglycerides and cholesterol. The differences in each of these conditions were primarily due to differing effects on peripheral tissue metabolism concerned with either uptake, utilization or degradation.

Publications: Amer. J. Clin. Nutr. 23:1069-1079, 1970;
Clin. Res. 19:45, 1971;
Program, Amer. Soc. Clin. Nutr., p. 3, 1971;
Fed. Proc. 30:348, 1971;
Program, Soc. Pediat. Res., p. 235, 1971.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 01: Pathogenesis of Infection of Military Importance

Work Unit No. 096 01 013: Host Lipids in Infectious and Toxic Illness

Description:

Study early changes in lipid metabolism during infectious and toxic illnesses.

Progress, Part I:

Although the body must depend chiefly on stored fat to meet its needs for energy during acute or severe infections, little is known concerning lipid metabolism during such illnesses[1/] (Amer. J. Clin. Nutr. 23:1069, 1970). Recent studies[2/] (New Eng. J. Med. 281:1081, 1969) which have shown increased mobilization of serum lipid during certain infections suggest that body lipids may respond in a somewhat different manner according to the etiologic agent involved.

Work has been completed on the first prospective study of serial measurement of serum lipids during experimentally induced infection using sandfly fever in volunteers. These studies (Medical Division Projects No. FY 70-3 and 71-1) were carried out as part of a cooperative study with Dr. Robert S. Lees of the Massachusetts of Institute of Technology.

Following hospitalization, 10 healthy young males were equilibrated for 17 days on a constant liquid diet containing 40% of total calories as fat. Blood lipid and lipoprotein concentrations were measured serially throughout the illness using methods previously described[3/] (USAMRIID Annual Progress Rep. FY 1969, p. 35). Quantitative lipid fractionating studies were carried out by ultracentrifugation by Dr. Lees.

After the equilibration period, 7 men were inoculated intravenously (iv) with sandfly fever virus and 3 with isotonic saline. No chemical or laboratory abnormalities occurred in sham-inoculated subjects. In the others, a typical brief, benign, febrile illness appeared 2.5 - 2.8 days after inoculation with virus. Fever, anorexia and reduced caloric intake contributed to an average weight loss of 5 pounds.

Biochemical changes appearing at the height of the illness included an increase in fasting blood sugar averaging 20 mg/100 ml above baseline control values and a decrease of 30 mg/100 ml in total plasma cholesterol. A reduction of similar magnitude occurred concomitantly in plasma phospholipid and triglyceride concentrations; the former remained low during convalescence and the latter increased above baseline control values. Additional changes became evident with the remission of fever; free fatty acids (FFA), cholesterol esters and plasma glycerol values declined, while β-hydroxybutyrate concentrations increased. Lactate and pyruvate levels were unchanged.

Lipoprotein electrophoresis revealed a late increase in the percentage of plasma pre-β (VLDL) lipoprotein with a corresponding decrease in the α (HDL) fraction. Ultracentrifugation lipoprotein analysis revealed a decline in the cholesterol content of the β (LDL) fraction which began during the incubation period.

Summary, Part I:

In volunteers infected with sandfly fever virus, altered patterns of lipid transport preceded the onset of clinical illness. A marked effect on both carbohydrate and lipid fuel stores was seen and varied with the stage of the infectious illness. The data are consistent with increased utilization and dependence on body fat stores for energy during this mild viral illness.

Progress, Part II:

Altered lipid responses occur in patients suffering severe bacterial disease.[2] The causes of the differing plasma lipid responses induced by gram positive and negative infections have yet to be explained. To explore the mechanism of such changes, sequential determinations were made of the lipid responses of rhesus monkeys to Diplococcus pneumoniae and Salmonella typhimurium infections. FFA metabolism was studied using ^{3}H-palmitic acid as previously described[4] (USAMRIID Annual Progress Rep. FY 1970, p.55).

Kinetic data were subjected to multicompartmental computer analysis.

The disappearance of labeled FFA from plasma and its incorporation into the triglyceride fraction in infected animals as compared to that in the noninfected control animals was increased. While increased plasma triglyceride concentrations developed in both infections, gram positive infections led to increases in plasma concentrations of FFA, phospholipid, and β lipoproteins and decreases in cholesterol. In contrast, gram negative infections led to decreased plasma concentrations of FFA, phospholipids, β lipoprotein and cholesterol.

Although the effect of both infections on FFA disappearance and triglyceride incorporation and concentrations were similar, the changes in serum lipid fractions were characteristic. This suggests that these infections differ in either their effect on uptake or utilization, or both, of lipid by peripheral tissue.

As hyperlipidemia during gram negative sepsis has largely been attributed to the effect of bacterial endotoxin, further work was done to determine if other infection related factors were involved. Lipid metabolism was studied during S. typhimurium septicemia or endotoxin induced by the toxin of the same species. Kinetic studies were performed.

Plasma triglyceride concentrations increased in both experimental groups. The rate of FFA disappearance from plasma and its subsequent incorporation into the triglyceride fraction increased in both the infection and endotoxemia. Plasma FFA concentrations were elevated during endotoxemia but were depressed during sepsis. During endotoxemia, IV administered glucose resulted in prolonged hyperglycemia, decreased disappearance rate of FFA and decreased incorporation of FFA into triglycerides. Similar glucose effects were not observed in Salmonella-infected monkeys.

Thus, acute gram negative septicemia induced changes in both fasted and glucose loaded monkeys which differed from that due to endotoxin. Similar kinetic data, in the face of differing plasma FFA concentrations suggest that the differences result from dissimilar effects on peripheral tissue metabolism that involve FFA uptake, its utilization, or both.

The experimental monkey model was expanded to examine more closely the dynamics of cholesterol metabolism during infection. The incorporation of ^{3}H-mevalonate into squalene (an intermediary product in cholesterol synthesis), cholesterol, and cholesterol ester was studied sequentially in fasted rhesus monkeys acutely infected with D. pneumoniae or S. typhimurium.

During infection with either bacterium, there was an increased rate of incorporation of the labeled mevalonate into plasma free cholesterol in comparison to values observed in fasted, normal control monkeys. When studied early in either infection, there was no evidence for an inhibition of squalene synthesis or its conversion into cholesterol. The estimated volume of cholesterol did not change, although total hepatic cholesterol did change. Although total hepatic cholesterol decreased significantly in Salmonella-infected monkeys, the specific activity of hepatic cholesterol did not differ from control values in either infection. Entry of radioactivity into plasma cholesterol ester was reduced in the Salmonella infection.

These data are suggestive of an increase in both synthesis and utilization of cholesterol during these acute bacterial infections, with more marked effect occurring during the gram negative infections.

Studies are in progress using the nonionic detergent WR 1339 which blocks the utilization of triglyceride by peripheral tissues. It is hoped that the mechanisms responsible for fuel utilization by peripheral tissues in both the fasted and fed states can be defined.

Summary. Part II:

The monkey model for infection and endotoxemia has been expanded; it enables us to examine more closely the dynamics of lipid metabolism during infection. These studies point to the importance of sequential determination of serum lipids and increased turnover of FFA, triglycerides and cholesterol during each state. The primary differences appear to be due to differing effects on peripheral tissue metabolism, uptake, utilization, or degradation or all of them.

Presentations:

1. Fiser, R. H. Host lipid response during infectious illness. Presented at Southern Society - American Federation for Clinical Research, New Orleans, La. 27-29 January 1971.

2. Fiser, R. H. Effect of acute infection on cholesterogenesis in the rhesus monkey. Presented at Annual Meeting of Federation of American Societies for Experimental Biology, Chicago, Ill. 12-17 April 1971.

3. Fiser, R. H. Sequential changes in plasma lipid and lipoproteins during sandfly fever. Presented at Annual Meeting American Society for Clinical Nutrition, Atlantic City, N. J. 1 May 1971.

Publications:

1. Beisel, W. R., and R. H. Fiser, Jr. 1970. Lipid metabolism during infectious illness. Amer. J. Clin. Nutr. 23:1069-1079.

2. Fiser, R. H., J. C. Denniston, and W. R. Beisel. 1971. Host lipid response during infectious illness. Clin. Res. 19:45 (abstract).

3. Fiser, R. H., J. C. Denniston, R. B. Rindsig, and W. R. Beisel. 1971. Effect of acute infection of cholesterogenesis in the rhesus monkey. Fed. Proc. 30:348 (abstract).

4. Fiser, R. H., J. C. Denniston, and W. R. Beisel. 1971. Gram negative septicemia versus endotoxemia: Differential effects on lipid metabolism. Society of Pediatric Research, Program and Abstracts p. 353 (abstract).

5. Fiser, R. H., R. S. Lees, W. R. Beisel, and P. J. Bartelloni. 1971. Sequential changes in plasma lipids and lipoproteins during sandfly fever. Amer. Soc. Clin. Nutr. Program p.3 (abstract).

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1. Beisel, W. R., and R. H. Fiser, Jr. 1970. Lipid metabolism during infectious illness. Amer. J. Clin. Nutr. 23:1069-1079.

2. Gallin, J. I., D. Kaye, and W. M. O'Leary. 1969. Serum lipids in infection. New Eng. J. Med. 281:1081-1086.

3. U. S. Army Medical Research Institute of Infectious Diseases, 1 July 1969. Annual Progress Report, FY 1969. p. 35 to 44. Fort Detrick, Maryland.

4. U. S. Army Medical Research Institute of Infectious Diseases, 1 July 1970. Annual Progress Report, FY 1970. p. 55 to 61. Fort Detrick, Maryland.

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ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 01:	Pathogenesis of Infection of Military Importance
Work Unit No. 096 01 014:	Development, Calibration and Standardization of Aerosol Equipment and Model Systems
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Division:	Animal Assessment
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Author:	Ralph W. Kuehne, B. S.
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

74

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA OL0909	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 01 29	D. CHANGE	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	01	014
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	CDOG 1212b(9)			

11. TITLE (Precede with Security Classification Code)*
(U) Development, Calibration and Standardization of Aerosol Equipment and Model Systems for Testing Military Vaccines (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*
003500 Clinical Medicine; 004900 Defense; 008700 Laboratories, Test Facilities

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
71 01	CONT	DA	C. In-house

17. CONTRACT GRANT
a. DATES/EFFECTIVE — EXPIRATION
b. NUMBER*
c. TYPE NA — d. AMOUNT
e. KIND OF AWARD — f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	1	8
	CURRENT 72	1	36

19. RESPONSIBLE DOD ORGANIZATION
NAME:* USA Medical Research Institute of Infectious Diseases
ADDRESS:* Fort Detrick, Md 21701
RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
NAME:* Animal Assessment Division USAMRIID
ADDRESS:* Fort Detrick, Md 21701
PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME:* Kuehne, R. W.
TELEPHONE: 301 663-4111 Ext 7244
SOCIAL SECURITY ACCOUNT NUMBER
ASSOCIATE INVESTIGATORS
NAME:
NAME:
DA

21. GENERAL USE
Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Aerosols; (U) Aerosol Generator; (U) Infectious Diseases; (U) Military Medicine

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Prepare specialized equipment for use in aerosol exposures of man and laboratory animals to biological materials and their products.

24 (U) Based upon specific requests and criteria, testing will be performed of all facets of aerosol exposure with the view of obtaining accurate and precise data.

25 (U) 70 07 - 71 06 - One hundred and one in vitro calibration runs were made with the modified Henderson apparatus using sodium fluorescein, Francisella tularensis and Diplococcus pneumoniae; a monkey aerosol exposure was conducted using D. pneumoniae. Good reliability and predictability were attained with little day-to-day variation.

One hundred and six in vitro calibration runs were made with the newly acquired standard Henderson apparatus using sodium fluorescein; a guinea pig aerosol exposure to bovine gamma globulin was conducted. Effective control of variables was accomplished, resulting in good reproducibility.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 01: Pathogenesis of Infection of Military Importance

Work Unit No. 096 01 014: Development, Calibration and Standardization of Aerosol Equipment and Model Systems

Description:

Prepare specialized equipment for use in aerosol exposures of man and laboratory animals to biological materials and their products.

Progress:

Background--In order to protect troops against the incapacitating effects of respiratory disease, a thorough understanding of the pathogenesis of the disease, as naturally acquired and transmitted, and the role of immunity must be accomplished. To ascertain this and to develop effective prophylaxis and therapy, animals and man must be experimentally exposed to definitive dosages of the organism by the natural port of entry, i.e., the respiratory route. Developed vaccines must also be evaluated periodically for protection against aerosol challenge to assure efficacy after prolonged storage prior to being administered to military personnel.

The aerosol equipment of the Institute is predominately used to support the work of other investigators. As a result, requests are often made for the aerosolization, exposure and assay of a wide variety of materials. For many of these materials, no previous experience is available, necessitating the development of model systems. This often requires determination of the optimal spray menstruum, the optimal recovery medium, the physical and biological decay rates, and the development of an assay system based on the most meaningful criteria to the investigator, e.g., viability, virulence, immunizing capacity, protein integrity, etc. In addition, *in vitro* calibration runs must be conducted to determine a spray factor in order to deliver exposure doses desired, even with materials for which a model system already exists. Calibration trials must also be performed each time the exposure apparatus is reassembled after sterilization and whenever a new stock culture is received. This work unit allows for the inclusion of data related to the development of systems and assays and calibration trials prior to actual requested exposures.

Part I:

Upon installation of the modified Henderson aerosol exposure apparatus in Animal Assessment Division Suite 5, 64 initial aerosol recovery trials were conducted with sodium fluorescein, utilizing a range of secondary air volumes (1-20 ft^3/min). A solution of sodium fluorescein in water for injection was disseminated at 80% relative humidity (RH), and the resultant aerosol sampled at one tube-sampling port with all-glass impingers (AGI-30), containing 20 ml water for injection and operating at 12.5 L/min for 10 min. Impinger fluids were assayed for dye content with a Photovolt fluorescence meter; for each trial, a spray factor was determined, which represents the logarithmic ratio of the concentration/ml of dye in the spray suspension to the concentration/L of dye recovered in the generated aerosol. The derived recovery curves resulted in the ability to predict recoveries in terms of amount of secondary air introduced into the system. This is a valuable technique which can be used to increase or decrease concentration in order to deliver a particular dosage without having to change the length of exposure or the concentration of the fill suspension.

Following these trials, a total of 40 recovery determinations were conducted at a constant secondary air input of 20 ft^3/min. For these trials, the human exposure panel was attached and sampling took place as previously, with one AGI-30 sampling directly off the tube and another from the exposure mask. The purpose of these trials was to determine reliability of tube-sampling during a volunteer exposure, to compare actual versus theoretical recoveries and to measure the degree of day-to-day variability. The theoretical physical spray factor was previously calculated to be about 3.55. Experimentally, the mean of 20 recoveries from the mask was 3.52, with a range of 3.44-3.59, and 20 tube recoveries averaged 3.50, ranging from 3.51-3.60. Recoveries were remarkably close to theoretical; there was no appreciable day-to-day variation and there was no significant difference between the tube-and mask-sampling points.

In preparation for an impending volunteer study, 56 calibration runs were made using Francisella tularensis, strain SCHU-S4. Fill suspensions were made in tryptose saline and aerosolized at 80% RH. AGI-30's contained 20 ml tryptose saline, plus one drop of Dow Corning antifoam A (DCA); fluids were plated on glucose cysteine blood agar. Sampling took place at the exposure mask and directly from the aerosol tube; there was good agreement between the 2 sampling points. Sixteen runs were conducted to establish recoveries at various airflows; the remaining 40 trials were all made at 20 ft^3/min. The mean spray factor of the latter 40 trials was 3.87, with a range of 3.66-4.25. This value is higher than the value obtained with sodium fluorescein, since the parameter of biological decay is added to that of physical decay. This compares favorably with recoveries obtained with F. tularensis when this apparatus was located in Bldg. 522 and indicates that good replication and predictability are feasible.

Prior to an exposure of Macaca mulatta to Diplococcus pneumoniae, Type I, 5 calibration runs were made to determine a spray factor. The

fill suspension consisted of 50 ml of an 18-hr culture in brain heart infusion broth plus 3 drops sheep blood plus 0.5 ml normal rabbit serum (NRS) plus 2 drops DCA. Impinger fluids consisted of 20 ml tryptose saline plus 2% NRS plus one drop DCA, and were plated on blood agar (5% sheep blood). The mean spray factor was 4.18 (range 4.11-4.33), indicating less aerosol stability than for F. tularensis. Six monkeys were subsequently exposed to D. pneumoniae for 10 min under similar conditions. Estimated presented doses ranged from 1.00-3.04 x 10^5, with a mean of 1.74 x 10^5.

Summary, Part I:

The modified Henderson apparatus has been installed in Bldg. 1425. One hundred and one in vitro calibration trials were conducted using sodium fluorescein, F. tularensis, and D. pneumoniae, and a monkey aerosol exposure was accomplished using D. pneumoniae. The apparatus showed good reliability, predictability and little day-to-day variation, with physical decay close to theoretical and biological decay comparable to that observed prior to relocation.

Progress, Part II:

A small (standard) Henderson aerosol exposure apparatus was received from the manufacturer and installed in the Class III biological safety cabinet system in AA Suite 4. Considerable modification was necessary before the apparatus was even in a workable condition and further modifications and additions (e.g., gauges, valves, etc.) were made to assure maximum efficiency and reproducibility. There were 106 calibration runs conducted with sodium fluorescein: 27 trials at various airflows and the remaining at a secondary air input of 16 L/min. Thirty of these latter trials were made using the guinea pig exposure tube, resulting in a mean spray factor of 2.73 logs, ranging from 2.65-2.80. When the monkey exposure box was substituted for the guinea pig tube, the mean spray factor of 49 trials was 2.82, with a range of 2.71-2.97. In both cases, the variation is about $\pm$ 0.1 log, which is considered to be indicative of excellent reproducibility and shows that good control of airflows, pressures, and other variables can be effectively maintained. Although the 0.1-log difference in the mean spray factor between the guinea pig tube and the monkey box is small, it can, nevertheless, probably be explained on the basis of the larger volume of the monkey box, resulting in a slightly greater dilution of the aerosol with a concomitant higher spray factor.

Following these trials, 36 Hartley guinea pigs were exposed to an aerosol of bovine gamma globulin (BGG) with sodium fluorescein added. Exposures of 20-min duration and impinger fluids were assayed for dye content, multiplied by the globulin:dye ratio and presented doses calculated for each group of 3 animals. A dose of 50-100 μg was desired. The original fill material consisted of 45 ml sterile water for injection, containing 14.05 mg/ml BGG and 10 μg/ml sodium fluorescein. Due to

foaming and clogging of the generator nozzle, the first group of 3 animals received 46.3 μg and the 2nd group received only 21.1 μg. The fill was then diluted to 7.02 mg/ml BGG plus 10 μg/ml fluorescein and the remaining 60 guinea pigs received doses from 59.3 μg to 80.2 μg, with a mean of 66.1 μg.

Summary, Part II:

A standard Henderson apparatus has been purchased and installed in the Class III system. One hundred and six *in vitro* calibration trials were conducted using sodium fluorescein; a guinea pig aerosol exposure to bovine gamma globulin was performed. Effective control of variable was attained, resulting in good reproducibility.

Publications:

None.

ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 01:	Pathogenesis of Infection of Military Importance
Work Unit No. 096 01 015:	Evaluation of Myocardial Contractility During Infectious Disease
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Division:	Physical Sciences
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Authors:	William H. Zech, Captain, VC John H. Boucher, Major, VC
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
	DA OL0911	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 04 20	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	01	015
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	CDOG 1212b(9)			

11. TITLE (Precede with Security Classification Code)* (U) Evaluation of Myocardial Contractility during Infectious Disease of Military Medical Importance (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*
003500 Clinical Medicine; 004900 Defense; 012900 Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
71 03	CONT	DA	C. In-house

17. CONTRACT/GRANT
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER*:
c. TYPE: NA d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.:

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	1	50
	CURRENT 72	1	71

19. RESPONSIBLE DOD ORGANIZATION
NAME:* USA Medical Research Institute of Infectious Diseases
ADDRESS:* Fort Detrick, Md 21701

RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
NAME:* Physical Sciences Division USAMRIID
ADDRESS:* Fort Detrick, Md 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME:* Zech, W. H.
TELEPHONE: 301 663-4111 Ext 7244
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Boucher, J. H.
NAME:
DA

21. GENERAL USE
Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Myocardium; (U) Physiology; (U) Infectious diseases; (U) Military Medicine

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Determine to what extent various infectious diseases cause changes in myocardial function.

24 (U) Using conventional methods of quantitating myocardial contractility, determine baseline values and then measure changes induced by infections induced in rhesus monkeys.

25 (U) 71 03 - 71 06 - Previous studies conducted under Work Unit 096 01 002 are reported here. Sotalol (a new beta-adrenergic receptor blocker) was found to be effective when given at a dose of 8.0 mg/kg to conscious dogs without myocardial depression. This effect was found to persist for at least 6 min following a 3-min infusion of Sotalol and atropine.

Increases in heart rate both by right atrial pacing and by Isoproteronal stimulation had no effect on myocardial contractility as measured by the V-max method.

Further studies have recently been started to determine normal baseline values for rhesus monkeys. When these values are obtained monkeys will be exposed to a model infection.

Publication: Fed. Proc. 30:228, 1971 (abstract).

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 01: Pathogenesis of Infection of Military Importance

Work Unit No. 096 01 015: Evaluation of Myocardial Contractility During Infectious Disease

Description:

Determine to what extent various infectious diseases cause changes in myocardial function.

Progress:

The initial 8 months work (previously carried under Work Unit 096 01 002) was devoted to surgical techniques, instrumentation, and project development of a study concerning the myocardial dynamics of thyrotoxic dogs. This study did not materialize due to the failure of such dogs to survive extensive amounts of instrumentation. Even after long recovery periods following surgery, dogs that were made thyrotoxic developed septicemia before experiments could be performed. Thyrotoxic dogs appeared to be much less capable of resisting infection than normal dogs. Due to these circumstances, a more productive project was sought and the present study was developed.

Assessment of the intrinsic myocardial functions, heart rate and myocardial contractility, during various stresses before and after autonomic blockage in conscious dogs was the primary objective. It was felt that by eliminating the heart's nervous supply, a sensitive evaluation of subcellular myocardial function could be obtained[1,2] (Amer. J. Cardiol. 24:198, 1969; Fed. Proc. 30:660, 1971).

Before this study could be initiated, there were important considerations which required 3 adjunctive studies. The first unknown was the optimum dose of Sotalol[R] (a new β-blocker produced by Mead Johnson Laboratories) to be combined with atropine; the second was the duration of effective blockade; and finally, the heart rate (HR) effects on contractility needed evaluation. These points were investigated in separate studies.

All studies were conducted on fully conscious dogs in which Dynasciences Model 1017 pressure microtransducers were implanted into the left ventricle at least 2 weeks earlier. They were trained to lie quietly on their sides while minimally restrained. Six to eight dogs per dose were used in the dose-response study, 8 dogs in the extent and duration study, and 6-8 dogs in the contractility-HR increment study. The dose of atropine that was combined with all Sotalol dosages was 0.2 mg/kg. The paired t test was used for significance determinations.

Myocardial contractility was measured by an estimation of the maximum velocity of contractile element shortening (Vmax)[3] (Amer. J. Cardiol. 26:248, 1970) using the developed pressure method which corrects for muscle pre-load[4] (Fed. Proc. 29:719, 1970). To compute this variable requires high fidelity left ventricular pressure (LVP) and its corresponding first derivative, "dp/dt', which is obtained by passing the pressure signal through an active, noninverting differential which then measures the rate of pressure development (figure 1). Recorder paper speed is 200 mm/sec. Mitral valve closure is instantaneous with the point where left ventricular end-diastolic pressure (LVEDP) is measured. Aortic valve opening (Po) occurs at the point on LVP so marked and is instantaneous with peak dp/dt. This point, Po, is "maximum isovolumic pressure". The period between LVEDP and Po is isovolumic contraction, during which time the ventricle is a closed chamber rapidly developing pressure. Contractile element velocity (Vce) is calculated at 5-msec intervals during the isovolumic period as (dp/dt)/32(DP). A pressure-velocity curve relating Vce to D^P is constructed. It was extrapolated by linear regression to 0 pressure to obtain an estimation of Vmax, measured in muscle length per second (ML/sec).

Table I presents data for determination of the adrenergic blockade induced by Sotalol + atropine (S + A) subsequently challenged with isoproterenol. A similar calculation is used for Vmax blockade.

TABLE I. EFFECT OF SOTALOL (8.0 mg/kg) and ATROPINE (0.2 mg/kg) ON HEART RATE CHANGE DUE TO ISOPROTERENOL, WITH RESULTING ESTIMATION OF CHRONOTROPIC BLOCKADE.

ISOPROTERENOL DOSAGE µg/kg	HEART RATE CHANGE (mean ± SEM) After S + A	Without S + A	% BLOCKADE[a] mean ± SEM
0.1		64 ± 4.2	
0.3	6 ± 4.2	97 ± 3.8	93 ± 3.1
1.0	19 ± 4.4	131 ± 3.8	86 ± 3.7
3.0	36 ± 4.1	147 ± 4.6	69 ± 6.3
10.0		166 ± 4.8	

a. $\% \text{ Blockade} = 100\left(1 - \dfrac{\text{HR change after S + A}}{\text{HR change without S + A}}\right)$

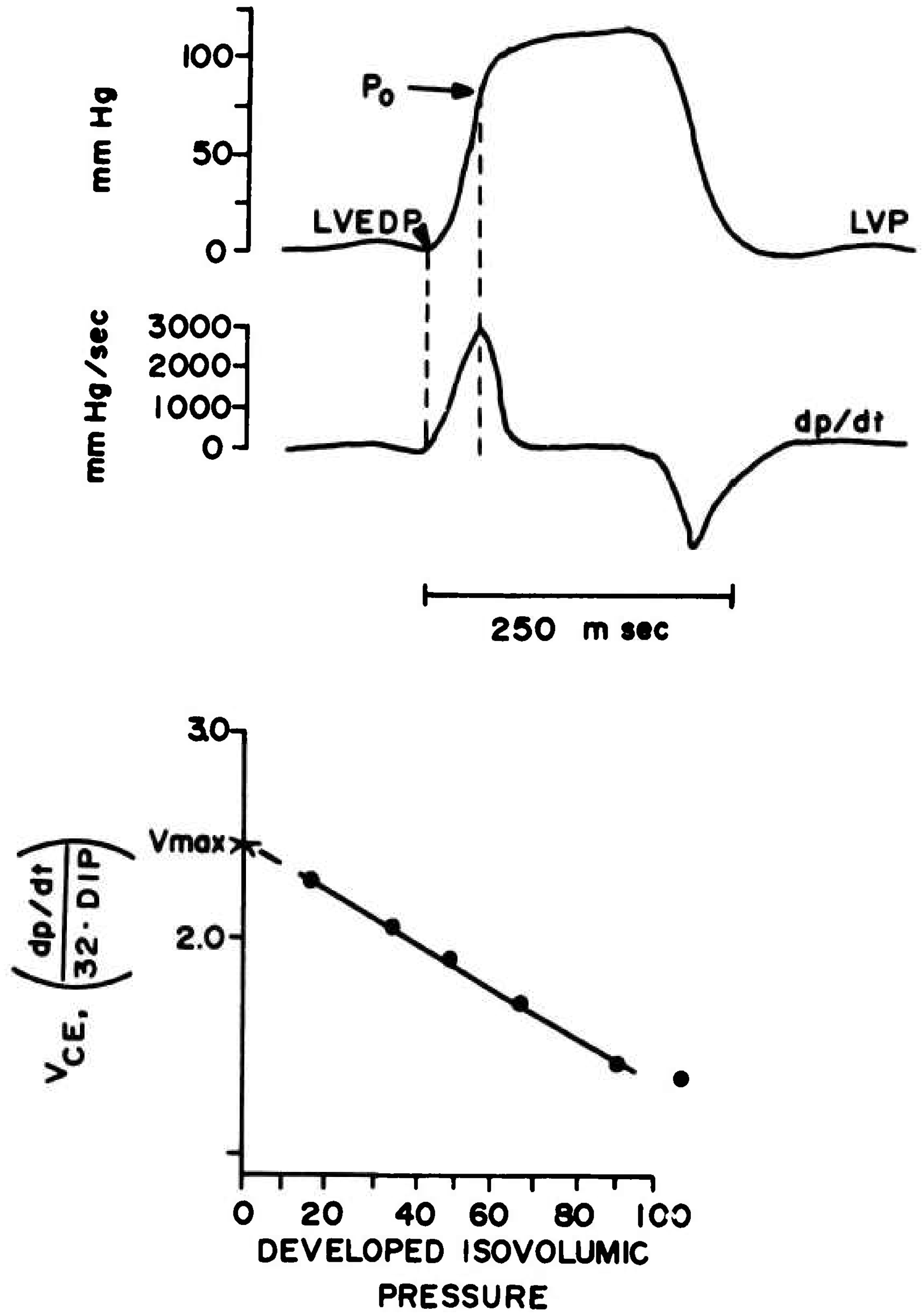

Figure 1. Estimation of Vmax.

Dose-response relationship for Sotalol--Sotalol, given in 6 logarithmically spaced doses, 0.5, 1.0, 2.0, 4.0, 8.0 and 16.0 mg/kg, was evaluated in this study. Each dog received no more than one dose every 48 hr. A baseline observation of the control group was followed by 5 isoproterenol stimulatory log doses, 0.1 to 10.0 μg/kg given at 10-min intervals. A dose level of S + A was given intravenously (IV) during a 3-min infusion. After a stabilization period of 7 min, an autonomic blockade baseline observation was made. This was followed by a repeat of the isoproterenol doses. The purpose of the isoproterenol administration was to change heart rate (HR) and myocardial contractility by selective -receptor stimulation in order to calculate percentage of chronotropic and inotropic blockade.

Isoproterenol was given as a bolus injection and exactly 25 sec later observations were made. Column 2 of Table I shows the effect of isoproterenol alone.

Dose-response curves were constructed for each isoproterenol dose level as the relationship between Sotalol combined with atropine sulfate and percentage chronotropic blockade (Table II).

TABLE II. TITRATION OF CHRONOTROPIC BLOCKADE BY ISOPROTERENOL GIVEN IN LOG DOSES

SOTALOL mg/kg + ATROPINE (0.2 mg/kg)	%BLOCKADE BY ISOPROTERENOL (μg/kg)				
	0.1	0.3	1.0	3.0	10.0
0.5	84 ± 2.1	50 ± 10.3	38 ± 4.2	34 ± 9.9	31 ± 6.9
1.0	85 ± 5.2	56 ± 8.2	43 ± 3.6	36 ± 5.9	29 ± 9.3
2.0	95 ± 1.8	84 ± 4.6	60 ± 4.1	51 ± 5.0	32 ± 7.6
4.0	96 ± 2.1	85 ± 3.5	70 ± 6.1	50 ± 6.0	32 ± 5.3
8.0		93 ± 3.1	86 ± 3.7	69 ± 6.3	
16.0		96 ± 1.8	93 ± 2.7	73 ± 4.9	

It can be seen that as Sotalol dosage increased there is greater blockade at the 4 lower doses of isoproterenol. At 10 μg/kg there is no change induced by Sotalol; two factors should be considered: one is the near-maximum stimulation caused by this dose and the second is the interaction of cholinergic blockade which is impossible to evaluate without further study.

When varying doses of Sotalol were combined with atropine and percentage change from control Vmax was determined, 16.0 mg/kg was the only dose which produced a significant percentage change from controls, indicating myocardial depression (Table III).

TABLE III. INOTROPIC BLOCKADE INDUCED BY VARYING DOSES OF SOTALOL WITH 0.2 mg/kg ATROPINE ON MYOCARDIAL CONTRACTILITY (Vmax in ML/sec)

SOTALOL mg/kg + ATROPINE	% CHANGE FROM CONTROL mean ± SEM	PROBABILITY P
0.5	- 14 ± 8.0	
1.0	9 ± 10.6	
2.0	4 ± 12.7	
4.0	- 6 ± 6.3	
8.0	- 4 ± 2.9	
16.0	- 11 ± 3.1	< 0.05

From these two titrations, 8.0 mg/kg Sotalol was chosen as optimal for the extent-and-duration-of-blockade study since it produced >90% chronotropic blockade to 0.3 μg/kg isoproterenol stimulation without myocardial depression. This was the lowest dosage level of Sotalol that satisfied the requirements and was chosen in spite of its high concentration, > 2.5 times the usual amount used experimentally in dogs.

Extent and duration of β-blockade in conscious, atropinized dogs--Baseline observations were obtained on 8 dogs; they were then stimulated with 0.3 μg/kg isoproterenol, for control values. This was followed by a 3-min IV infusion of 8 mg/kg Sotalol combined with atropine sulfate. After a stabilization period of 10 min an autonomic blockade baseline observation was made. This was followed by 6 repeat isoproterenol challenges of 0.3 μg/kg at 10 min intervals from 10-60 min.

Th control isoproterenol stimulation increased the heart rate 74% and Vmax 38% from the baseline values. The extent of chronotropic blockade (HR) was greater than inotropic blockade (Vmax) and was relatively constant at about 85%, whereas, inotropic blockade was more erratic throughout the 60 min (Table IV).

TABLE IV. EXTENT AND DURATION OF AUTONOMIC BLOCKADE ON HR AND Vmax

TIME min	% BLOCKADE (mean $\pm$ SEM) HR	Vmax
+10	87 $\pm$ 10.4	76 $\pm$ 12.8
+20	92 $\pm$ 2.4	86 $\pm$ 11.9
+30	87 $\pm$ 7.5	76 $\pm$ 16.6
+40	86 $\pm$ 4.7	55 $\pm$ 19.2
+50	76 $\pm$ 8.2	64 $\pm$ 18.6
+60	83 $\pm$ 8.6	59 $\pm$ 12.3

Neither the chronotropic nor the inotropic mean blockade values at any of the time periods were significantly different from the mean blockade value at 10 min, indicating that effective and relatively constant blockade was maintained for the entire time.

The heart rate following a 3-min infusion of S + A was presumed to be the intrinsic rate. The mean value for all time periods from 0-10 min was significantly greater than the mean control rate of 114 $\pm$ 9.9 beats/min (Table V). The extent and duration of the intrinsic rate was stabilized at 145 beats/min at 30 min, gradually falling to about 132 beats/min at the end of 60 min.

TABLE V. INTRINSIC HEART RATE FOLLOWING AUTONOMIC BLOCKADE

TIME min	BEATS/MIN Mean (+ SEM)
Control	114 ± 9.9
0	145 ± 11.6
1	146 ± 8.8
2	144 ± 9.3
3	147 ± 11.2
4	147 ± 10.8
5	144 ± 11.9
6	147 ± 10.7
7	147 ± 11.6
8	152 ± 12.8
9	148 ± 11.6
10	146 ± 12.0

From the dose-response and the extent-and-duration studies it was concluded that Sotalol, in the high dose used, produced an effective β-blockade which persisted for at least 60 min, but did not have myocardial depressant activity when administered in combination with atropine to conscious dogs.

Quantitation of myocardial contractility with increments in HR-- Recent reports are contradictory regarding the influence of HR on myocardial contractility. All studies found in the literature in which contractility was measured in anesthetized patient[5,6/] (Circulation 33:945, 1966 and 42 Suppl. III):45, 1970) or animals[7,8/] (Amer. J. Physiol. 205:30, 1963; Cardiovasc. Res. 1:2, 1967), or isolated cardiac muscle[9-11/] (Saechs. Akad. Wiss. Leipzig. Ber. 23:652, 1871; Fed. Proc. 21:975, 1962; Circulation 42 (Suppl. III):163, 1970) showed that heart rate had a positive influence on contractility. In the only two papers found in which dogs were conscious, heart rate was shown not to have an influence on contractility[12,13/] (Circ. Res. 19:206, 1966; 24:285, 1969). The purpose of this study was to assess chronotropic inotropism in order to separate the two influences on myocardial contractility, as measured by Vmax.

The heart was paced by means of an impulse generation connected to an electrode implanted surgically in the right atrial epicardium. Heart rate was increased by 10-20 beats/min from the control rate to slightly more than 200 beats/min. Bolus injections of 5 isoproterenol log doses (0.03, 0.1, 0.3, 1.0, and 3.0 μg/kg) were given IV at 3-10 min intervals. This resulted in a graded increase in HR to ~ 250 beats/min. Isoproterenol challenges were repeated following a 3-min infusion of Sotalol + atropine. S + A produced both β-adrenergic and cholinergic blockade and allowed study of the intrinsic myocardial response to increases in heart rate. During these HR pacing studies there was no correlation between frequency of contraction and Vmax either before or after autonomic blockade. There was, however, a significant correlation between HR and Vmax ($\underline{P} < 0.05$), without autonomic blockade (Table VI) produced by isoproterenol.

TABLE VI. CORRELATION OF HEART RATE AND Vmax INDUCED BY ISOPROTERENOL

HR beats/min	Vmax ML/sec	REGRESSION ANALYSIS
93	2.01	
129	2.51	
129	2.26	
132	2.44	
135	2.19	
138	2.34	
138	2.39	
147	2.61	
150	2.45	
153	2.61	
153	2.63	
156	2.43	
160	2.20	
165	2.45	
184	2.38	
186	2.49	$r = 0.52$
190	3.59	
192	2.31	
192	2.47	$\hat{Y} = 1.634 + 0.006X$,
195	2.77	
198	3.00	where $\hat{Y} = a + bX$
201	3.66	
204	2.56	$SEM_b = 0.002$
210	2.39	
210	2.60	
216	3.45	
222	2.58	
222	2.68	
223	2.73	
234	2.74	

A correlation between HR and Vmax did not occur during isoproterenol dose increments after autonomic blockade, in spite of the fact that HR increased > 40 beats/min. Thus, the contractile state of the unblocked myocardium was augmented by isoproterenol, but after autonomic blockade the inotropic effects were inactive and the positive chronotropism did not influence contractility. This verifies that the absence of chronotropic inotropism during heart rate pacing was not artifactual. It was concluded that HR does not have an influence on the myocardial contractile state in conscious dogs.

Future studies will utilize the rhesus monkeys (Macaca mulatta) in order to determine if similar quantitation of myocardial contractility, is possible and practical in the intact, conscious animal.

Summary:

Sotalol (a new β-adrenergic receptor blocker) was found to be effective, with no myocardial depression when given at a dose of 8.0 mg/kg to conscious dogs. This effect was found to persist for at least 60 min following a 3-min IV infusion of Sotalol and atropine.

Increases in heart rate both by right atrial pacing and by isoproterenol stimulation had no effect on myocardial contractility as measured by the Vmax method.

Presentations:

Boucher, J. H. Effective β-adrenergic receptor blockade with Sotalol in the absence of myocardial depression. Presented at Federation of American Societies for Experimental Biology, Chicago, Ill. 12-17 April 1971.

Publication:

Boucher, J. H., W. H. Zech, and A. L. Stagg. 1971. Effect of β-adenergic receptor blockade with Sotalol in the absence of myocardial depression. Fed. Proc. 30:228 (abstract).

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ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 01:	Pathogenesis of Infection of Military Importance
Work Unit No. 096 01 105:	Capillary Ultrastructure in Bacterial Infection
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Division:	Pathology
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Author:	Thomas H. Hudson, Captain, MSC
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA OL0905	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISB'N INSTR'N	8b. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
70 12 22	D. CHANGE	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	01	105
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	CDOG 1212b(9)			

11. TITLE (Precede with Security Classification Code)*
(U) Capillary Ultrastructure in Bacterial Infections of Military Medical Importance (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*
010100 Microbiology; 004900 Defense; 002600 Biology (Pathology)

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
70 12	CONT	DA	C. In-house

17. CONTRACT/GRANT
a. DATES/EFFECTIVE: | EXPIRATION:
b. NUMBER*: NA
c. TYPE: | d. AMOUNT:
e. KIND OF AWARD: | f. CUM. AMT.:

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	1	5
	CURRENT 72	1	47

19. RESPONSIBLE DOD ORGANIZATION
NAME:* USA Medical Research Institute of Infectious Diseases
ADDRESS:* Fort Detrick, Md 21701
RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
NAME:* Pathology Division USAMRIID
ADDRESS:* Fort Detrick, Md 21701
PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME:* Hudson, T. H.
TELEPHONE: 301 663-4111 Ext 2134
SOCIAL SECURITY ACCOUNT NUMBER:
ASSOCIATE INVESTIGATORS
NAME:
NAME: DA

21. GENERAL USE
Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Military Medicine; (U) Bacterial diseases; (U) Pathogenesis; (U) Molecular biology; (U) Circulatory system

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Investigate ultrastructure and permeability changes of capillaries resulting from bacterial infection.

24 (U) Infect white mice, introducing protein markers at varying times; study the tissues ultrastructurally.

25 (U) 70 12 - 71 06 - Infection of mice with Diplococcus pneumoniae is seen to produce changes in the capillary permeability to tracer molecules, ferritin and horseradish peroxidase. These changes occur during the first 4 hr of infection. Of the tissues prepared for electron microscopy (lung, liver, muscle, and intestine), only liver and muscle have been examined. Between 4 and 9 hr postinfection, muscle capillaries do not seem to change their permeability to the tracers. During the same time interval, liver capillaries show a marked increase in permeability to the tracer proteins. Continuing malfunctions of the RCA EMU-3F electron microscope have hampered progress.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 01: Pathogenesis of Infection of Military Importance

Work Unit No. 096 01 105: Capillary Ultrastructure in Bacterial Infection

Description:

Investigate ultrastructure and permeability changes of capillaries resulting from bacterial infection.

Progress, Part I:

The ultrastructural basis for capillary permeability has been studied using exogenous, water soluble protein tracers (either horseradish peroxidase with a molecular diameter of approximately 500 nm or ferritin, 1100 nm in normal tissues. Studies performed on normal liver[1/] (Lab. Invest. 20:298, 1969), muscle[2/] (J. Cell Biol. 37:277, 1968), and gut[3/] (J. Cell Biol. 41:33, 1969), indicate the existence of several regional differences in the permeability of blood capillaries to these large protein molecules. The authors also demonstrated several mechanisms for the movement of water soluble proteins through capillary walls. The requirements of various organs for serum proteins remain relatively constant in the healthy organism. However, during the course of an infection these priorities are shuffled. The permeability of the capillaries to serum proteins during an infection must also be changed. It is the ultrastructure of this change which is the subject of this study.

The development of a technique for the ultrastructural localization of horseradish peroxidase (HRP) and ferritin in Diplococcus pneumoniae-infected mice was examined. A variety of fixatives were tried, with both good tissue fixation and preservation of enzymatic peroxidase activity being the criteria for choosing the best. A combination of gluteraldehyde and formaldehyde[4/] (J. Histochem. Cytochem. 14:291, 1966), gave the best fixation while retaining HRP activity. Because of the high osmolality of this fixative (approximately 2000 milliosmols), tissues removed from infected mice and placed directly into this solution showed varying degrees of cell destruction. This drawback was overcome by injecting 8 ml of the cold fixative into the peritoneum of anesthetized animals. After 3 min the animals were opened and tissues removed, placed in modified (diluted by half) Karnovsky's fixative.[4/] Fixation was carried out at 0 C for 3-4 hr. The tissues were washed in buffer overnight. HRP (3-5 mg in 0.5 ml of saline) or ferritin (90 mg) was injected through the tail vein of normal mice. These animals were sacrificed from 0.5-60 min after introduction of the tracer proteins. After HRP injection, 1.25 min was chosen as the best time for fixation to observe the passage of the protein across the capillary wall.

After ferritin injection, 3 min was considered most advantageous.

After fixation, as described above, the tissues were cut into 50-100 μ sections on the cryostat. The slices were incubated in the Graham-Karnovsky medium[4] (0.05 M Tris-HCl buffer, pH 7.6, 10 ml, containing 0.01% H_2O_2 and 10 mg of 3-3'-diaminobenzidine tetrahydrochloride) then washed 3 times in distilled water and postfixed in O_sO_4 and processed for electron microscopy. Tissues from ferritin-injected animals were processed as above except for incubation in the peroxidase medium.

Summary, Part I:

A satisfactory method for fixation and ultrastructural localization of HRP and ferritin in normal and Diplococcus infected mice has been developed

Progress, Part II:

A series of mice were injected intraperitoneally with 6 D. pneumoniae, Type I, organisms. Two animals each at 4, 8, 12, 24, and 36 hr postinfection were injected intravenously with HRP and sacrificed 1.25 min later. Intestine, liver, muscle, and lung were prepared as described but only liver and muscle were examined. During the entire time range of the experiment the amount of HRP in the space of Disse of infected mouse liver was greater than control mice. The movement of HRP across infected muscle capillaries did not show a substantial change from the control mice.

Summary, Part II:

The liver capillaries increase their permeability to HRP some time during the first 4 hr after infection, and maintain this increase through 9 hr of infection. Muscle capillaries do not seem to change their permeabilities to HRP.

Progress, Part III:

A series of mice were injected with ferritin and HRP simultaneously and fixed 1-3 min after injection. After processing for HRP localization, the ultrastructure of liver and muscle was studied. Initial observations show HRP passing through the capillary wall at a greater rate than ferritin.

Summary, Part III:

Passage of protein through capillary walls seems to be related to the size of the molecule.

Publications:

None.

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3. Clementi, F., and G. E. Palade. 1969. Intestinal capillaries. I. Permeability to peroxidase and ferritin. J. Cell Biol. 41:33-58.

4. Graham, Jr., R. C., and M. J. Karnovsky. 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney. Ultrastructural cytochemistry by a new technique. J. Histochem. Cytochem. 14:291-302.

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ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 01:	Pathogenesis of Infection of Military Importance
Work Unit No. 096 01 401:	Effect of Bacterial and Viral Infections on Host Cell Biosynthetic Mechanisms
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Division:	Physical Sciences
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Authors:	Michael C. Powanda, Captain, MSC, (I) Robert W. Wannemacher, Jr., Ph.D., (I) William L. Steinhart, Captain, MSC, (II)
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
	DA [illegible]0809	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 01 06	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A0[illegible]	01	401
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1212b(9)			

11. TITLE (Precede with Security Classification Code)* (U) Effect of Bacterial and Virus Infections on Host Cell Biosynthetic Mechanisms in BW Defense Research (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS* 002300 Biochemistry; 003500 Clinical Medicine; 004900 Defense

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
62 10	CONT	DA	C. In-house

17. CONTRACT/GRANT
a. DATES/EFFECTIVE: | EXPIRATION:
b. NUMBER:*
c. TYPE: NA | d. AMOUNT:
e. KIND OF AWARD: | f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	2	30
	CURRENT 72	2	116

19. RESPONSIBLE DOD ORGANIZATION
NAME:* USA Medical Research Institute of Infectious Diseases
ADDRESS:* Fort Detrick, Md 21701

RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
NAME:* Physical Sciences Division USAMRIID
ADDRESS:* Fort Detrick, Md 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME:* Powanda, M. C.
TELEPHONE: 301 663-4111 Ext 5214
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Wannemacher, Jr., R. W.
NAME: Steinhart, W. S. DA

21. GENERAL USE
Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Rats; (U) Mice; (U) Pneumococcus; (U) Tryptophan metabolism; (U) Liver; (U) Chromatin; (U) Military Medicine

23. TECHNICAL OBJECTIVE.* 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Study alterations in nucleotide metabolism, RNA biosynthesis, and template activity of isolated chromatin in host cells during infection.

24 (U) Total nicotine-adenine dinucleotide (NAD) is measured in the tissues of mice subjected to a variety of conditions including infection with pneumococcus. The incorporation of radioactivity labeled precursors of RNA into RNA isolated from various subcellular fractions is measured in the host during infection.

25 (U) 70 07 - 71 06 - There were no significant changes in hepatic TO activity, tryptophan content or pyridine nucleotide concentrations during the early stages of pneumococcal infection in the rat. Once, however, bacteremia becomes well developed, there were increases in TO activity and liver tryptophan. The latter observation coupled with the demonstration of an increased flux of amino acids from muscle and liver during pneumococcal sepsis in the rat, strongly suggest that the increased urinary excretion of tryptophan metabolites seen in infection of various etiologies result from increased availability of tryptophan rather than from elevated TO activity.

The early rise in liver chromatin template activity of pneumococcus infected mice is absent in adrenalectomized animals indicating the need for intact adrenals for the host to respond. Liver chromatin isolated 5 hr after pneumococcus infection has an increased capacity to hold actinomycin D, further evidence that there is a functional alteration in transcription during infection.

Publications: J. Nutr. 100:1471-1478, 1970
Biochim. Biophys. Acta 228:301-305, 1971
Fed. Proc. 30:232, 517, 1971
Proc. Soc. Exp. Biol. Med. 137:619-622, 1971

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 01: Pathogenesis of Infection of Military Importance

Work Unit No. 096 01 401: Effect of Bacterial and Viral Infections on Host Cell Biosynthetic Mechanisms

Description:

Study alterations in tryptophan metabolism and nucleic acid synthesis in animals during infection.

Progress, Part I:

A study of the urinary metabolites of volunteers with experimentally induced sandfly fever, typhoid fever, tularemia or Rocky Mountain spotted fever indicated that the catabolism of tryptophan via the kynurenine pathway was increased irrespective of the etiology of the illness and appeared to be correlated with the extent of fever[1/] (J. Infect. Dis. 122: 159, 1970). In order to explore the mechanism of this generalized host response, animal models were selected. Mice infected with *Diplococcus pneumoniae* exhibited a marked but transient rise in hepatic tryptophan oxygenase (TO) activity during the first 24 hr after infection; it was thus suggested that the increase in enzyme activity brought about the increased catabolism of tryptophan[2/] (Arch. Intern. Med. 121:11, 1968). However, because of the controversy as to whether TO activity[3/] (J. Clin. Invest. 45:1527, 1966) or tryptophan availability[4/] (J. Biol. Chem. 244: 1410, 1969) regulates the functioning of the kynurenine pathway in normal animals, fundamental studies had to be conducted before the effect of infection itself could be ascertained. Only the liver was analyzed for TO activity and tryptophan content because this tissue contains the preponderant amount of TO activity[5/] (J. Biol. Chem. 240:1395, 1965). Nicotinamide adenine dinucleotide (NAD) which can be formed in the liver from tryptophan[6/] (J. Biol. Chem. 238:3369, 1963) was chosen as an index of tryptophan catabolism rather than urinary metabolites, because of its highly significant role in both synthetic and degradative metabolism.

Dietary studies conducted in rats[7/] (J. Nutr. 100:1471, 1970) indicated a high degree of correlation between liver cell NAD concentration and the tryptophan content of the diet. Because evening values for TO activity also demonstrated a correlation with intake of tryptophan, this approach did not provide an answer as to whether enzyme activity or substrate availability was the controlling factor. Further studies making use of pulse doses of tryptophan alone or in combination with agents which would either lower or raise TO activity had to be carried out[8/] (Fed. Proc. 30:232, 1971). Mice were given single intraperitoneal (IP) injections of

varying amounts of tryptophan (75-500 mg/kg) in saline. After this injection there was an increase in hepatic tryptophan content within minutes which was quickly followed by a rapid rise in TO activity and an augmentation of NAD somewhat later (2-3 hr). All of these changes were proportional to the amount of tryptophan administered. Allopurinol, an inhibitor of the TO system, given either simultaneously or 1 hr before tryptophan decreased enzyme activity from 35 to 70% without markedly affecting the concentration of NAD. A pharmacologic dose of hydrocortisone almost tripled oxygenase activity without noticeably increasing NAD content.

The conclusion to be drawn from both the dietary and pulse dose experiments is that the NAD concentration in liver is both responsive to and regulated by the availability of substrate, under normal conditions. By extrapolation one might presume that all tryptophan metabolism via kynurenine is also so regulated.

With these fundamental experiments completed, studies were then conducted in rats infected with _D. pneumoniae_. Infection was accomplished by the subcutaneous injection of 10^6 pneumococci. Controls were given a similar volume of heat-killed organisms. All animals were fasted after inoculation. Fever was apparent by 14 hr and maximal at 28. Bacteremia was evident by 14 hr, became massive by 28 hr and persisted thereafter. Control animals displayed a diurnal variation in hepatic tryptophan content, TO activity and NAD concentration. Infected animals, however, did not display such rhythmicity in either NAD concentration or enzyme activity and this accounts in part for some of the alterations seen in these entities when compared to control values.

There were no significant changes in hepatic TO activity, tryptophan content or pyridine nucleotide concentrations (NAD, NADH, NADP, NADPH) during the early stages of pneumococcal infection. Once, however, bacteremia became well developed, there was an increase in TO activity and a rise in liver tryptophan concentration. Thus the increased urinary excretion of tryptophan metabolites seen in infections of various etiologies could result from an increased availability of tryptophan as well as from elevated TO activity. Recent observations which have demonstrated an increased flow of amino acids from muscle to tissue such as liver during pneumococcal infection (manuscript in preparation) lend further support to the suggestion that is the availability of tryptophan which regulates its catabolism.

There was also a transient increase in hepatic NAD 24 hr postinfection which does not appear to emanate from the enhanced TO activity since the rate of NAD synthesis from a standard dose of L-tryptophan was the same in both control and infected animals.

Whether or not the above changes are an intentional part of host defense or merely indicators of a generalized host response cannot, at the moment, be ascertained.

Summary, Part I:

By manipulating the dietary tryptophan content of rats and by the use of pulse doses of tryptophan in mice, alone and in combination with agents which will alter TO activity, evidence was amassed indicating that the hepatic NAD concentration was both responsive to and regulated by the availability of tryptophan rather than by alterations in TO activity. There were no significant changes in hepatic TO activity, tryptophan content or pyridine nucleotide concentration during the early stages of pneumococcal infection in the rat. Once bacteremia was well developed, there was an increase in TO activity and a rise in liver tryptophan. The latter observations coupled with the demonstration of an increased flux of amino acids from muscle to liver during pneumococcal sepsis in the rat, strongly suggest that the increased urinary excretion of tryptophan metabolites seen in infection of various etiologies results from increased availability of tryptophan rather than from elevated TO activity.

Progress, Part II:

The assay of liver chromatin template activity in our generalized infection model of D. pneumoniae in mice has suggested an involvement of gene transcription in the early response of the liver[9,10] (Fed. Proc. 30:517, 1971; Proc. Soc. Exp. Biol. Med., 137:619, 1971). The early increase in template activity has been found to be absent in adrenalectomized, infected mice.[9,10] Intact adrenal glands also appear to be essential for the full, nocturnal increase normally found in control mice[11] (Biochim. Biophys. Acta 228:301, 1971). Mice starved for 29 hr prior to injection with pneumococci still maintain the nocturnal, rhythmic increase,[11] demonstrating that the infection-related decrease in chromatin template activity at 14 hr is not due simply to self-imposed starvation among the sick animals.

The binding of ^{3}H-actinomycin D to isolated chromatin was used as an assay of unrestricted chromosomal regions[12] (Arch. Biochem. Biophys., 138: 272, 1970). Examination of the binding capacity of chromatin isolated 5 hr postinfection showed that binding increased over that of controls. These results support our earlier hypothesis that more sites on the liver chromatin are available for transcription very early during the infectious process.

Techniques for the electrophoretic analysis of ribonucleic acid species have been worked out. Using ^{32}P labeling and polyacrylamide gel electrophoresis, we hope to define in size and functional class the RNA molecules whose synthesis is stimulated early in infection.

Summary, Part II:

The early rise in liver chromatin template activity of pneumococcus-infected mice is absent in adrenalectomized animals. Starvation does not eliminate the normal, nocturnal rise in chromatin template activity.

Adrenalectomy partially obliterates it. Liver chromatin isolated 5 hr after pneumococcus injection has an increased capacity to bind the drug actinomycin D.

Presentations:

1. Powanda, M. C. Relationship between tryptophan oxygenase activity and hepatic nicotinamide adenine dinucleotide concentration. Presented at Annual Meeting, Federation of American Societies for Experimental Biology, Chicago, Ill. 12-17 April 1971.

2. Steinhart, W. L. Role of the chromatin template during protein synthesis in mouse liver. Presented at Annual Meeting, Federation of American Societies for Experimental Biology, Chicago, Ill. 12-17 April 1971.

Publications:

1. Powanda, M. C., and R. W. Wannemacher, Jr., 1970. Evidence for a linear correlation between the level of dietary tryptophan and hepatic NAD concentration and for a systematic variation in tissue NAD concentration in the mouse and the rat. J. Nutr. 100:1471-1478.

2. Steinhart, W. L. 1971. Diurnal rhythmicity in template activity of mouse liver chromatin. Biochim. Biophys. Acta 228:301-305.

3. Powanda, M. C., and R. W. Wannemacher, Jr. 1971. Relationship between tryptophan oxygenase activity and hepatic nicotinamide adenine dinucleotide concentration. Fed. Proc. 30:232 (abstract).

4. Steinhart, W. L. 1971. Role of the chromatin template during protein synthesis in mouse liver. Fed. Proc. 30:517 (abstract).

5. Steinhart, W. L. 1971. Alteration of template activity of chromatin from livers of pneumococcus-infected mice. Proc. Soc. Exp. Biol. Med. 137: 619-622.

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1. Rapoport, M. I., W. R. Beisel, and R. B. Hornick. 1970. Tryptophan metabolism during infectious illness in man. J. Infect. Dis. 122: 159-169.

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[illegible]. Altman, K., and O. Greengard. 1966. Correlation of kynurenine [illegible]ion with liver tryptophan pyrrolase levels in disease and after hydrocortisone induction. J. Clin. Invest. 45:1527-1534.

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7. Powanda, M. C., and R. W. Wannemacher, Jr. 1970. Evidence for a linear correlation between the level of dietary tryptophan and hepatic NAD concentration and for a systematic variation in tissue NAD concentration in the mouse and the rat. J. Nutr. 100:1471-1478.

8. Powanda, M. C., and R. W. Wannemacher, Jr. 1971. Relationship between tryptophan oxygenase activity and hepatic nicotinamide adenine dinucleotide concentration. Fed. Proc. 30:232 (abstract).

9. Steinhart, W. L. 1971. Role of the chromatin template during protein synthesis in mouse liver. Fed. Proc. 30:517 (abstract).

10. Steinhart, W. W. 1971. Alteration of template activity of chromatin from livers of pneumococcus-infected mice. Proc. Soc. Exp. Biol. Med. 137: 619-622.

11. Steinhart, W. L. 1971. Diurnal rhythmicity in template activity of mouse liver chromatin. Biochim. Biophys. Acta 228:301-305.

12. Beato, M., K. H. Seifart, and C. E. Sekeris. 1970. The effect of cortisol on the binding of actinomycin D to and on the template activity of isolated rat liver chromatin. Arch. Biochem. Biophys. 138:272-284.

ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents(U)
Task No. 1B662711A096 01:	Pathogenesis of Infection of Military Importance
Work Unit No. 096 01 403:	Host-parasite Relationships in Pathogenesis of Virus Infections
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Division:	Animal Assessment
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Authors:	Richard O. Spertzel, Lt Colonel, VC Donald E. Kahn, Captain, VC Carroll L. Crabbs
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION: DA 0L0812
2. DATE OF SUMMARY: 71 07 01
REPORT CONTROL SYMBOL: DD-DR&E(AR)636

3. DATE PREV SUM'RY: 71 02 12
4. KIND OF SUMMARY: D. CHANGE
5. SUMMARY SCTY: U
6. WORK SECURITY: U
7. REGRADING: NA
8a. DISB'N INSTR'N: NL
8b. SPECIFIC DATA-CONTRACTOR ACCESS: [X] YES [ ] NO
9. LEVEL OF SUM: A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	01	403
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1212b(9)			

11. TITLE (Precede with Security Classification Code): (U) Host-parasite Relationships in Pathogenesis of Virus Infections of Military Medical Importance (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical Medicine; 004900 Defense; 002600 Biology

13. START DATE: 61 07
14. ESTIMATED COMPLETION DATE: CONT
15. FUNDING AGENCY: DA
16. PERFORMANCE METHOD: C. In-house

17. CONTRACT/GRANT
- a. DATES/EFFECTIVE:
- EXPIRATION:
- b. NUMBER: NA
- c. TYPE:
- d. AMOUNT:
- e. KIND OF AWARD:
- f. CUM. AMT.:

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	1	30
	CURRENT 72	2	15

19. RESPONSIBLE DOD ORGANIZATION
- NAME: USA Medical Research Institute of Infectious Diseases
- ADDRESS: Fort Detrick, Md 21701
- RESPONSIBLE INDIVIDUAL
- NAME: Crozier, D.
- TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
- NAME: Animal Assessment Division USAMRIID
- ADDRESS: Fort Detrick, Md 21701
- PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
- NAME: Spertzel, R. O.
- TELEPHONE: 301 663-4111 Ext 7244
- SOCIAL SECURITY ACCOUNT NUMBER:
- ASSOCIATE INVESTIGATORS
- NAME: Kahn, D. E.
- NAME:

DA

21. GENERAL USE: Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Encephalitis (VEE, EEE); (U) Virus diseases; (U) Arboviruses; (U) Yellow fever; (U) Teratology; (U) Military Medicine

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Identify and study factors involved in the pathogenesis of virus diseases.

24 (U) Extend investigations of the effect of arboviruses on the fetus, in order to understand how the observed effect on fetuses occurs, e.g. in the placenta, etc.

25 (U) 70 07 - 71 06 - Work was resumed late in the reporting period. Studies are only preliminary at this time and will not be reported until next fiscal year.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 01: Pathogenesis of Infection of Military Importance.

Work Unit No. 096 01 403: Host-parasite Relationships in Pathogenesis of Virus Infections

Description:

Identify and study factors involved in the pathogenesis of virus diseases.

Progress:

The outcome of viral infection is dependent upon a number of variables. The host-parasite relationship may be expressed as clinical disease, subclinical, inapparent infection, or as latent infection. This relationship involves reactions between host cell populations on the one hand and virus populations on the other.

This work unit is a continuing project which has been under active investigation for more than 7 years. Present efforts are directed toward finalizing earlier work on the transplacental transmission of live, attenuated Venezuelan equine encephalomyelitis virus vaccine (a tissue culture strain in the 83rd passage level) and its effect on the fetuses of pregnant mice.

Studies were initiated recently to determine relative viremia levels in the dams and their fetuses when the former were inoculated with virus on the 10th day of gestation. Previous studies had shown the maximum detrimental effect on the fetuses and newborn to occur when dams were inoculated at this time of gestation[1/] (CES Annual Report, FY 1970, p. 275). Additional studies, to determine whether the attenuated virus acts directly on the fetuses or the placentae to produce the observed results, are planned in the near future.

Summary:

Work was resumed late in the 4th quarter of the year. Studies are preliminary at this time and will not be reported until next fiscal year.

Publications:

None.

LITERATURE CITED

1. Commission on Epidemiological Survey. Dec 1970. Annual Report FY 1970 to the Armed Forces Epidemiological Board. p. 275-281. Fort Detrick, Maryland.

ANNUAL PROGRESS REPORT

Project No. 3B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 01:	Pathogenesis of Infection of Military Importance
Work Unit No. 096 01 404:	Mouse Brain Ultrastructure in Viral Infections
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Division:	Pathology
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Author:	Hilary Evans, Major, MC
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

110

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
	DA OL0906	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISB'N INSTR'N	8b. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
70 12 22	D. CHANGE	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	01	404
b. CONTRIBUTING				
~~c. CONTRIBUTING~~	CDOG 1212b(9)			

11. TITLE (Precede with Security Classification Code)*
(U) Mouse Brain Ultrastructure in Viral Infectionsof Military Medical Significance (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*
010100 Microbiology; 004900 Defense; 002600 Biology (Pathology)

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
70 12	CONT	DA	C. In-house

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER*: NA
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	1	5
	CURRENT 72	1	78

19. RESPONSIBLE DOD ORGANIZATION

NAME:* USA Medical Research Institute of Infectious Diseases
ADDRESS:* Fort Detrick, Md 21701

RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION

NAME:* Pathology Division
USAMRIID
ADDRESS:* Fort Detrick, Md 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME:* Evans, H.
TELEPHONE: 301 663-4111 Ext 2134
SOCIAL SECURITY ACCOUNT NUMBER

ASSOCIATE INVESTIGATORS
NAME: Hudson, T. H.
NAME: DA

21. GENERAL USE

Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Encephalomyelitis, equine (VEE); (U) Pathogenesis; (U) Molecular biology; (U) Nervous System; (U) Military Medicine

23. TECHNICAL OBJECTIVE.* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Determine the ultrastructure of VEE virus infection in the mouse brain to gain insight into host response and pathogenesis.

24 (U) Mice infected with VEE virus were killed at regular time intervals and their brains examined by electron microscopy.

25 (U) 70 12 - 71 06 - Twenty-four litters of suckling mice have been infected and their brains prepared for electron microscopy. Thus far, all phases of the replicative cycle of the virus have been identified with the electron microscope.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 01: Pathogenesis of Infection of Military Importance

Work Unit No. 096 01 404: Mouse Brain Ultrastructure in Viral Infections

Description:

Study the ultrastructure of VEE viru ection of the mouse brain.

Progress:

Background - The ultrastructure of ian equine encephalomyelitis (VEE) virus has been studied in tissue culture cells and in preparations of isolated virus but not in the intact animal. The purpose of this investigation is to examine the virus within neurons, in all stages of its replicative cycle, and to note the changes in the host cells. The information gained should provide insight into the pathogenesis of the encephalitis caused by this virus.

In order to see this virus in tissue with the electron microscope, one must attain a titer of 10^7 median mouse intracerebral lethal doses ($MICLD_{50}$) per gram of tissue. As this is not possible in the adult mouse, suckling mice were used. Twenty-four litters were injected intraperitoneally with 1000 LD_{50} of Trinidad strain VEE virus. They were killed at 12-hr intervals through 48 hr (when all surviving mice were moribund). At 24, 36, and 48 hr the mouse brains had viral titers higher than 10^7 weanling $MICLD_{50}$/gm tissue. The brains have been processed for electron microscopy.

In preliminary work on mice treated this way, all phases of the viral replicative cycle were observed. Technical difficulty with the electron microscope has thus far prevented the examination of this tissue.

Publications:

None.

ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 01:	Pathogenesis of Infection of Military Importance
Work Unit No. 096 01 800:	Biological Effects of Microbial Toxins
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Divisions:	Animal Assessment, Bacteriology and Pathology
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Authors:	Joseph C. Denniston, Captain, VC (I, II) Michael D. Kastello, Captain, VC (II) James W. Stiles, Captain, MSC (I) Virginia G. McGann, Ph.D. (I, II) Douglas W. Mason, Captain, VC (II) Richard O. Spertzel, Lt Colonel, VC (II)
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OL0871	71 07 01	DD DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 01 06	D. CHANGE	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	01	800
b. CONTRIBUTING				
c. ~~xxxxxxx~~	CDOG 1212b(9)			

11. TITLE (Precede with Security Classification Code)
(U) Biological Effects of Microbial Toxins of Military Medical Importance (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
016800 Toxicology; 003500 Clinical Medicine; 002300 Biochemistry; 004900 Defense

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
66 10	CONT	DA	C. In-house

17. CONTRACT/GRANT
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER:
c. TYPE: NA
d. AMOUNT:
e. KIND OF AWARD:
f. CUM. AMT.:

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	3	40
	CURRENT 72	3	60

19. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, Md 21701
RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
NAME: Animal Assessment Division USAMRIID
ADDRESS: Fort Detrick, Md 21701
PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Kastello, M.D.
TELEPHONE: 301 663-4111 Ext 7244
SOCIAL SECURITY ACCOUNT NUMBER
ASSOCIATE INVESTIGATORS
NAME: Mason, D. W.
NAME: Spertzel, R. O. DA

21. GENERAL USE
Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Staphylococcus; (U) Enterotoxin; (U) Chronic infusion; (U) Lung pathology; (U) Leukocytes; (U) Military Medicine

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Study the biological effects of microbial toxins.

24 (U) The effects of staphylococcal enterotoxin B (SEB) on animal hosts are measured by a number of parameters.

25 (U) 70 07 - 71 06 - Monkeys continuously infused with SEB exhibited marked differences from bolus-injected monkeys. While both groups developed leukopenia, the leukopenia persisted throughout the experimental period in the continuously infused animals, but the single-bolus-injected animals demonstrated a rebound leukocytosis. Pulmonary endothelial cell necrosis was both characteristic for, and more severe in, the continuously infused animals. Severity of lesions varied directly with dose of infused toxin.

Preliminary work for study of per os-induced intoxication of monkeys with microbial toxins is nearly complete.

Publication: Amer. J. Vet. Res. 31:1845-1851, 1970.

*Available to contractors upon originator's approval

DD FORM 1498 MAR 68 — PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 01: Pathogenesis of Infection of Military Importance

Work Unit No. 096 01 800: Biological Effects of Microbial Toxins

Description:

Study the biological effects of microbial toxins in the animal host.

Progress, Part I:

A preliminary report was presented last year[1/] (USAMRIID Annual Progress Report, FY 1970, p. 75) concerning the continuous infusion of staphylococcal enterotoxin B (SEB). It was anticipated that the continuous infusion methods employed would resemble the continuous release of toxin that may occur during staphylococcal sepsis of man and produce a sustained exposure of all tissues to the toxin. This report summarizes the results of the study.

The concentrations of SEB in blood produced by continuous infusion of toxin could be assessed only at the largest challenge dose (5 mg/kg/5 hr), since the concentration at the smaller dose levels was below sensitivity limits of the toxin assay procedure. Although serum SEB was undetectable in these animals at 30-min time, a total serum concentration of 0.5 mg was obtained following a 2-hr infusion of 6 mg SEB. At the end of the 5-hr infusion, total serum SEB reached the 0.7-mg level.

Almost all toxin-challenged animals developed classical signs of SEB intoxication, commencing 1-2 hr following the start of the infusion. These clinical signs included anorexia, retching, emesis, diarrhea, pyrexia, listlessness, pale mucous membranes and death.

The clinical hematology and blood chemistry values of monkeys infused with SEB are shown in Table I. A significant ($\underline{P} < 0.01$) leukopenia, as compared to prechallenge control values, developed within 2 hr of the time when infusion was initiated, and persisted throughout the experimental period. All leukocyte cell types were involved. Immature cells of the granulocytic series began to appear in small numbers 4 hr following the start of the infusion, but no significant increase was noted in their number. A significant ($\underline{P} < 0.05$) decrease in hematocrit values, paralleling the changes in leukocyte counts, was observed during the infusion studies.

A slight increase in blood urea nitrogen (BUN) was observed. Significant ($\underline{P} < 0.05$) elevation in serum glutamic oxalacetic transaminase (SGOT) values occured 30 hr after commencing the infusion. A significant

TABLE I. CLINICAL HEMATOLOGY AND CHEMISTRY VALUES DURING SEB INFUSION ± 1 SE 15 μg/kg/30 HOURS IN 4 MONKEYS

TIME (HOURS)	WBC x 10^3	PCV %	BUN (mg/100 ml)	SGOT (UNITS)	PROTEIN (g/100 ml)
Prechallenge	17.10 ± 1.44	35.2 ± 0.8	18 ± 1.3	43.2 ± 5.6	6.1 ± 0.2
0.5	15.77 ± 2.31	32.7 ± 0.5 a/			
1	13.66 ± 1.75	30.2 ± 1.3 b/			
2	8.23 ± 1.62 b/	30.2 ± 1.5 a/			
4	5.34 ± 1.84 c/	29.8 ± 1.2 b/			
6	8.69 ± 2.08 b/	31.7 ± 0.8 a/	20 ± 1.1	46.2 ± 5.5	5.7 ± 0.2
12	7.08 ± 0.52 c/	32.0 ± 1.7	20.7 ± 1.9	52.5 ± 5.5	5.2 ± 0.2 a/
24	6.62 ± 1.33 c/	29.5 ± 0.8 c/	27.2 ± 4.6	66.7 ± 5.5	4.8 ± 0.2 b/
30	d/	d/	31.2 ± 5.9	74.0 ± 6.5 a/	4.7 ± 0.2 b/
36	d/	d/	33.7 ± 6.4	77.0 ± 6 a/	4.9 ± 0.2 b/

a. $P < 0.05$.
b. $P < 0.01$.
c. $P < 0.001$.
d. Samples lost due to laboratory accident.

decrease ($\underline{P} < 0.01$) in plasma proteins developed progressively during the study.

Postmortem examination of intoxicated animals revealed acute pulmonary hemorrhage and congestion. The monkeys infused with 5 mg/kg/5 hr developed the most severe lesions. Histologic lesions consisted primarily of acute perivascular, peribronchial and intraalveolar edema that included a mixed cell population of alveolar macrophages, lymphocytes, neutrophils and erythrocytes. Control monkeys presented no evidence of disease when necropsied at the end of 60 hr of saline infusion.

Electron micrographs of control lungs showed pulmonary capillaries with endothelial linings that extended without interruption about the capillary walls. Type I pneumocytes, however, were observed to have occasionally undergone a process of rounding up into small vesicles that remained close to the intact basement membrane.

Monkeys that died from small-dose SEB infusions presented a characteristic pulmonary picture. Degenerating leukocytes were distributed throughout the tissue; some interstitial edema was noted. The most significant finding was that endothelial cells of pulmonary capillaries at some locations were pinched off and rounded into vesicles. Transudate and erythrocytes were noted in some alveolar spaces.

Although endothelial cell breakdown was seen at all SEB infusion levels, the type of damage encountered served to characterize the dose level. Vesicular rounding up of endothelial cells characterized the small-dose levels, while endothelial cell swelling and fragmentation were characteristic of large-dose levels. In either case, distribution of lesions was very patchy. The 30-nm, dark-staining, round bodies were frequently observed in damaged capillaries and were characteristic of the endothelial lesion; they occasionally occurred with endothelial breakdown, simultaneous vesiculation of Type I pneumocytes which created totally unbounded, but intact, basement membranes. Capillary necrosis was also present in close proximity to ruptured leukocytes that had released their vacuoles into the capillary lumen. Large numbers of leukocytes were frequently observed in pulmonary tissue of all SEB-treated monkeys.

The experimental approach to the study of SEB intoxication used in this report produced results clearly different from those obtained by other experimental designs. The 2 monkeys administered single-bolus injections of SEB exhibited classical signs of SEB intoxication and presented the typical SEB-related pulmonary lesions previously described. Continuously infused SEB monkeys also exhibited classical signs of intoxication. but, in addition, showed 2 distinct differences. First, while both groups developed leukopenia, the leukopenia persisted throughout the experimental period in continuously infused animals, whereas rebound leukocytosis was observed in single-bolus-injected animals. Second, pulmonary endothelial cell necrosis was both characteristic for, and more severe in, the continuously infused monkeys. Additionally, severity of

the lesion increased in proportion to doses of infused toxin.

The fact that persistent leukopenia is observed in toxin-infused monkeys, while early leukopenia followed by a rebound leukocytosis is seen in bolus-injected animals, suggests that either SEB is not recirculated following its initial clearance, or that it is present in only trace amounts, or as an altered, undetected molecule. The leukopenia observed during infusion studies, which involved all leukocytic cell types, could have resulted from the following 3 mechanisms: impaired release of leukocytes, their destruction, and their sequestration in tissues.

Impaired production or release is suggested by the lack of a rebound leukocytosis and the absence of immature cells of the granulocytic series during the infusion period. In single-bolus-challenge experiments with SEB, rebound leukocytosis developed 3-6 hr postchallenge. Immature cells of the granulocytic series reach 90% of the total population by the 9th hr following challenge. Impaired release of leukocytes alone would not seem sufficient to account for the relatively rapid development of the initial leukopenia observed during the infusion studies. However, impaired production or release of leukocytes could account in part for the persistence of the leukopenia.

Sequestration of leukocytes into various tissues could account for their early, rapid removal from the general circulation. Large numbers of leukocytes were observed in the pulmonary tissues of continuously SEB-infused animals. This sequestration of leukocytes in pulmonary capillaries may result from toxin-leukocyte membrane interactions which render the leukocytes sticky, thus causing them to adhere to endothelial cells. On the other hand, an SEB endothelial cell membrane interaction might render the endothelial cells sticky, resulting in trapping of leukocytes within capillary beds. Still, the toxin itself may serve as a catalyst for the production of a leukopenia-producing substance. Whatever the mechanism of leukocyte sequestration, the principle site of leukocyte-trapping appears to be in pulmonary capillaries, since similar sequestration of leukocytes was not observed in hepatic, splenic or renal tissues during this study.

Leukocyte destruction as a contributing factor in the initial early leukopenia seems unlikely. However, disrupted white cells were observed in pulmonary capillaries and could contribute to the sustained leukopenia during continuous toxin infusion. It thus seems that leukocyte sequestration would account for the initial rapid leukopenia observed, and that sequestration, impaired release, and destruction of leukocytes all contribute to the sustained leukopenia observed throughout the infusion studies.

The small (30 nm), dark-staining bodies observed in pulmonary capillaries of the intoxicated monkeys have not been previously described and may be indicative of severe endothelial damage by SEB. Since these small bodies are observed in both intact endothelial cells and leukocytes, their release may be a result of a cytopathic effect of the toxin, resulting in cellular disruption.

A close relationship probably exists between leukocytes, leukopenia, leukocyte disruption, leukocyte sequestration and endothelial cell damage. As previously noted in this study, leukocytes were frequently observed adjacent to areas of pulmonary endothelial cell necrosis, but less frequently in intact areas. Since both ruptured and intact leukocytes were noted at points of endothelial breakdown, it is uncertain whether intact leukocytes are secreting necrotizing substances into capillaries at these points, or even whether ruptured cells are participating through the release of their lytic cellular organelles into the capillary lumen.

The increased severity, but not occurrence, of the electron microscopic lesions produced by infusions of high doses of SEB (1 mg/kg/hr) suggests that SEB is involved in the necrotic process itself.

A manuscript has been submitted for clearance to publish results of this work.

Summary, Part I:

Monkeys continuously infused with SEB exhibited marked differences from bolus-injected monkeys. While both groups developed leukopenia, the leukopenia persisted throughout the experimental period in the continuously infused animals, but the single-bolus-injected animals demonstrated a rebound leukocytosis. Pulmonary endothelial cell necrosis was both characteristic for, and more severe in, the continuously infused animals. Severity of lesions varied directly with dose of infused toxin.

Progress, Part II:

Increased emphasis is being placed on the physiology and mechanism of *per os*-induced intoxication with microbial toxins. This is due, in part, to a reorientation of the mission of the Institute. In addition, the continuing problem of food poisoning in troops, complicated by the paucity of physiologic data, has provided the stimulus.

To approach this problem, it is necessary to establish a physiologic model for tracking the toxin molecule after acute and chronic exposure via the alimentary tract. This requires surgical implantation of specific cannulation devices at selected anatomical sites within the gastrointestinal tract to facilitate both infusion of the toxin and sampling of the gastrointestinal contents in the conscious animal.

Permanent cannulation devices have been developed for use in the dog and these must be extensively modified before they are suitable for use in the monkey.

Summary, Part II:

Preliminary work for study of *per os* induced intoxication of monkeys with microbial toxins is nearly complete.

Publication:

Rhoda, D. A., and W. R. Beisel. 1970. Lymph production during staphylococcic B enterotoxemia - induced shock. Amer. J. Vet. Res. 31:1845-1851.

LITERATURE CITED

1. U. S. Army Medical Research Institute of Infectious Diseases. 1 July 1970. Annual Progress Report, FY 1970. p. 75 to 84. Fort Detrick, Maryland.

ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 01:	Pathogenesis of Infection of Military Importance
Work Unit No. 096 01 801:	Mediators of Microbial Toxin Activity
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Division:	Physical Sciences
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Author:	Anthony C. Jung, Captain, MSC
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
	DA OL0872	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 04 15	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	01	801
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1212b(9)			

11. TITLE (Precede with Security Classification Code)*

(U) Mediators of Microbial Toxin Activity in BE Defense Research (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

016800 Toxicology; 003500 Clinical Medicine; 004900 Defense

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
66 10	CONT	DA	C. In-house

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER*: NA

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	2	50
	CURRENT 72	1	70

19. RESPONSIBLE DOD ORGANIZATION

NAME:* USA Medical Research Institute of Infectious Diseases

ADDRESS:* Fort Detrick, Md 21701

RESPONSIBLE INDIVIDUAL

NAME: Crozier, D.

TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION

NAME:* Physical Sciences Division USAMRIID

ADDRESS:* Fort Detrick, Md 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME:* Jung, A. C.

TELEPHONE: 301 663-4111 Ext 6130

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME:

NAME: DA

21. GENERAL USE

Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Military Medicine; (U) Bradykinin; (U) Kinin system; (U) Radioimmunoassay; (U) Hypotension

23. TECHNICAL OBJECTIVE.* 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Evaluate the role of host mediators in the action of microbial toxins.

24 (U) A radioimmunoassay technique is employed to determine bradykinin plasma levels in animals which have been infected or intoxicated with agents know to have hemodynamic manifestations.

25 (U) 70 07 - 71 06 - Difficulties in preparing suitable labeled hapten and anti-bradykinin antisera have delayed the utilization of a radioimmunoassay technique to explore the involvement of bradykinin in infection.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 01: Pathogenesis of Infection of Military Importance

Work Unit No. 096 01 801: Mediators of Microbial Toxin Activity

Description:

Evaluate the role of host mediators in the action of microbial toxins.

Progress:

Bradykinin is a vasoactive polypeptide found in plasma which produces vasodilatation and subsequent hypotension. Tella and Maegnaith[1] (Trans. Roy. Soc. Trop. Med. 56:6, 1962) studied a *Plasmodium* infection in monkeys and found a decrease in levels of the bradykinin precursor, bradykininogen. Other investigators studying intoxications with either *Escherichia coli* or its endotoxin found that a vasoactive polypeptide was released[2] (Surg. Gynec. Obstet. 118:807, 1964), that there was a rise in levels of plasma kinin and a decrease in plasma kininogen[3] (Circ. Res. 22:155, 1968), that the rise in plasma kinin levels could be prevented by a protease inhibitor with coinciding decreases in fatalities[4] (Arch. Immun. Ther. Exp. 17:406, 1969), that a kinin was released *in vitro* in human plasma when exposed to *E. coli* or its endotoxin[5] (Life Sci. 9:313, 1970). Other workers have found no change[6] (Experientia 26:63, 1970) or the opposite effect[7] (Fed. Proc. 27:92, 1968) in similar studies. Recent human studies have shown that in gram negative bacteremia there is a definite increase in liberation of bradykinin, as evidenced by changes in levels of factors in the plasma kinin system[8,9] (Clin. Res. 17:371, 1969; Ann. Intern. Med. 71:763, 1969). However, this increase was seen only in bacteremic patients with associated hypotension. Normotensive patients had normal levels of the measured factors.

To investigate bradykinin involvement in certain infections, a suitable assay technique was necessary. The method of choice is the radioimmunoassay technique recently perfected by Talamo and his group[10] (J. Lab. Clin. Med. 74:816, 1969). It is specific, simple and sensitive to the 0.01-ng level.

Progress in setting up the bradykinin radioimmunoassay has been somewhat slowed. First, after two attempts, an apparently successful radioiodination of tyrosyl-8-bradykinin was performed. High radioactivity was present in the eluants resulting from passage of the reaction mixture through a column of Dowex 1. After diluting this labeled hapten to the desired activity, attempts were made to determine the antibody titer in the

antiserum collected from the immunized rabbits. Most undiluted samples demonstrated binding activity, but as the antisera were diluted the desirable bound to free ratio of 1:1 - 3:1 could not be attained. When Talamo and his associates performed a preliminary analysis of some of these antisera, they demonstrated a definite antibody response also, but they examined only undiluted samples. Undoubtedly two possibilities exist: (1) an inadequate antibody response in the immunized rabbits or (2) a poorly labeled and/or impure labeled hapten. In regard to the first possibility, the following paths are now being explored: (1) immunization of 2 additional rabbits with immunogen previously prepared, (2) preparation of fresh immunogen and injection into additional rabbits, and (3) possible use of additional tests, e.g. a micro-complement fixation test and determination of binding affinity, to examine previously harvested antisera. To investigate the second possibility, a portion of the labeled hapten form the second iodination attempt was again passed through an anion exchange column in hope of removing any undesirable labeled products which might be present. However, use of the labeled hapten eluted from the column gave similarly poor results when tested with antisera. Therefore, another attempt was made to carry out a successful radioiodination. The labeled hapten which resulted seems promising in that it binds well to antisera and very poorly to control sera. However, none of the antisera harvested up to this point demonstrates an adequate binding capacity. An attempt will be made to obtain from Dr. Talamo a sample of the antiserum he uses in his radioimmunoassay and see how well our hapten binds to it. When these problems with the hapten and antisera are finally resolved, the assay technique can then be utilized in exploring our stated objective.

Summary:

Difficulties in preparing suitable labeled hapten and anti-bradykinin antisera have delayed the utilization of the radioimmunoassay technique to explore the involvement of bradykinin in infection.

Publications:

None

LITERATURE CITED

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2. Kobold, E. E., R. Lovell, W. Katz and A. P. Thal. 1964. Chemical mediators released by endotoxin. Surg. Gynec. Obstet., 118:807-813.

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10. Talamo, R. C., E. Haber, and K. F. Austen. 1969. A radioimmunoassay for bradykinin in plasma and synovial fluid. J. Lab. Clin. Med. 74:816-827.

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ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 01:	Pathogenesis of Infection of Military Importance
Work Unit No. 096 01 802:	*In vivo* Distribution of Microbial Toxins
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Division:	Pathology
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Authors:	Joseph F. Metzger, Colonel, MC Anna D. Johnson
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
	DA OL0873	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 04 15	D. CHANGE	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	01	802
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1212b(9)			

11. TITLE (Precede with Security Classification Code)
(U) In vivo Distribution of Microbial Toxins of Military Medical Importance (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
003500 Clinical Medicine; 004900 Defense; 016800 Toxicology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
66 10	CONT	DA	C. In-house

17. CONTRACT GRANT
a. DATES EFFECTIVE — EXPIRATION
b. NUMBER
c. TYPE NA — d. AMOUNT
e. KIND OF AWARD — f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	1	40
	CURRENT 72	1	50

19. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, Md 21701

RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
NAME: Pathology Division USAMRIID
ADDRESS: Fort Detrick, Md 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Metzger, J. F.
TELEPHONE: 301 663-4111 Ext 6206
SOCIAL SECURITY ACCOUNT NUMBER

ASSOCIATE INVESTIGATORS
NAME:
NAME: DA

21. GENERAL USE
Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Enterotoxin; (U) Staphylococcus; (U) Immunofluorescence; (U) Immunoenzymatic techniques; (U) Isotopic tracers; (U) Antigen; (U) Antibody; (U) Military Medicine

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Study the transport and localization of microbial toxins in animal tissues and the pathogenesis of the corresponding intoxications.

24 (U) Use an in vivo isotopically labeled enterotoxin and study the pharmacology utilizing this preparation.

25 (U) 70 07 - 71 07 - Isotopically labeled enterotoxin has been prepared and preliminary in vivo studies have been accomplished. The in vivo labeling permits the identification of either the toxin or its breakdown products by either scintillation or radioautography.

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 01: Pathogenesis of Infection of Military Importance

Work Unit No. 096 01 802: In vivo Distribution of Microbial Toxins

Description:

Study transport and localization of microbial toxins in animal tissues and the pathogenesis of the corresponding intoxications.

Progress:

Various *in vitro* labeled staphylococcal enterotoxin B (SEB) preparations have been utilized to determine the dynamic pharmacology of enterotoxin B[1-3/] (J. Clin. Invest. 45:1365, 1966; J. Infect. Dis. 117:273, 1967; J. Bact. 93:779, 1967). SEB has been labeled with ^{131}I, ^{125}I, fluorescein, peroxidase, and tritium by utilizing *in vitro* techniques. *In vitro* labels separate either spontaneously or through the actions of enzymes when these *in vitro* labeled toxins are tested in animals. Fluorescein and ^{131}I are both separated almost completely when the toxin is administered via the gastrointestinal tract. Therefore, the labeled toxin has in no way elucidated toxin adsorption, distribution, or fate by this route. By the intravenous and intrapulmonary routes a similar displacement of the label occurs and most measurements have been made utilizing trichloroacetic acid (TCA) precipitation as an indication of that portion of the label which is still attached to SEB. It has been demonstrated by all routes that ^{125}I or ^{131}I can attach *in vivo* to proteins other than SEB. Therefore a portion of the TCA precipitable activity may well represent free ^{125}I or ^{131}I which has attached to proteins other than the toxin and previous localization studies may represent either the toxin or an extraneous protein.

Personnel of U. S. Army Biological Laboratories[4/] (Technical Manuscript 464), demonstrated an *in vivo* labeling SEB with the isotope ^{14}C. This material was purified by the method of Schantz et al.[5/] (Biochemistry 4:1011, 1965), and the activity was verified by immunological and toxicological methods.

Tritium labeled enterotoxin B was prepared by growing the organism *Staphylococcus aureus* strain 10-275 in a medium containing tritiated N-Z Amine (Wilzbach procedure). The enterotoxin was purified by a modification of the method of Schantz et al.[5/] Levels of incorporation have varied from 5-400 cpm/µg of purified SEB. Further studies are being continued to increase the incorporation of the radioactive isotope.

A preliminary study was conducted, in which 2 mg/kg of ^{3}H-SEB was inoculated intravenously into a rhesus monkey. Clearance from the blood was apparently complete before the 80-min sampling (Table I).

TABLE I. BLOOD LEVELS OF ^{3}H-SEB IN A RHESUS MONKEY

TIME POSTINOCULATION min	CPM	SEB LEVEL µg/ml
20	54	1.2
40	26	0.6
60	14	0.3
80	0	0
120	0	0

The animal was euthanitized at 120 min. Various organs were tested for labeled toxin (Table II).

TABLE II. TISSUE LEVELS OF ^{3}H-SEB IN A RHESUS MONKEY

ORGAN	CPM/100 mg
Kidney, left	1084
right	724
Liver	450
Spleen	111
Lung	40
Heart	30

Summary:

Tritiated SEB was prepared utilizing a ^{3}H N-Z Amine as a portion of the culture media. Purification was carried out according to a modified method of Schantz.[57] Preliminary testing *in vivo* was done.

Publications:

None.

LITERATURE CITED

1. Rapoport, M. I., L. F. Hodoval, E. W. Grogan, V. McGann, and W. R. Beisel. 1966. The influence of specific antibody on the disappearance of staphylococcal enterotoxin B from blood. J. Clin. Invest. 45:1365-1372.

2. Morris, E. L., L. F. Hodoval, and W. R. Beisel. 1967. The unusual role of the kidney during intoxication of monkeys by intravenous staphylococcal enterotoxin B. J. Infect. Dis. 117:273-284.

3. Rapoport, M. I., L. F. Hodoval, and W. R. Beisel. 1967. Influence of thorotrast blockade and acute renal artery ligation on disappearance of staphylococcal enterotoxin B from blood. J. Bact. 93:779-783.

4. Bowden, J. P. June 1968. A method for preparing small quantities of C^{14}-labeled staphylococcal enterotoxin B. Technical Manuscript 464. Biological Sciences Laboratory, Fort Detrick, Maryland.

5. Schantz, E. J., W. G. Roessler, J. Wagman, L. Spero, D. A. Dunnery, and M. S. Bergdoll. 1965. Purification of staphylococcal enterotoxin B. Biochemistry 4:1011-1016.

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ANNUAL PROGRESS REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 01: Vulnerability of Man to Biological Agents

Work Unit No. 096 01 803: Subcellular Biological Effects of Microbial Toxins and Microbial Disease

Reporting Installation: U. S. Army Medical Research Institute of Infectious Diseases
Fort Detrick, Maryland

Divisions: Bacteriology and Pathology

Period Covered by Report: 1 July 1970 to 30 June 1971

Professional Authors: Peter G. Canonico, Captain, MSC (I, II, III, V)
Matthew J. Van Zwieten, Captain, VC (I, II, III, V)
James W. Stiles, Captain, MSC (IV)

Reports Control Symbol: RCS-MEDDH-288(R1)

Security Classification: UNCLASSIFIED

134

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
	DA OL0896	71 07 01	DD DR&E(AR)636

3. DATE PREV SUMMARY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 01 06	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	01	803
b. CONTRIBUTING				
c. ~~[illegible]~~	CDOG 1212b(9)			

11. TITLE (Precede with Security Classification Code)* (U) Subcellular Biological Effects of Microbial Toxins of Military Medical Importance (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*
003500 Clinical Medicine; 016800 Toxicology; 002300 Biochemistry; 004900 Defense

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
69 07	CONT	DA	C. In-house

17. CONTRACT/GRANT
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER:*
c. TYPE: NA d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	2	40
	CURRENT 72	2	69

19. RESPONSIBLE DOD ORGANIZATION
NAME:* USA Medical Research Institute of Infectious Diseases
ADDRESS:* Fort Detrick, Md 21701
RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
NAME:* Bacteriology and Pathology Divisions USAMRIID
ADDRESS:* Fort Detrick, Md 21701
PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME:* Canonico, P. J.
TELEPHONE: 301 663-4111 Ext 7341
SOCIAL SECURITY ACCOUNT NUMBER:
ASSOCIATE INVESTIGATORS
NAME: Van Zwieten, M.
NAME: DA

21. GENERAL USE
Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Enterotoxin; (U) Staphylococcus; (U) Lysosomes; (U) Enzymes; (U) Pulmonary endothelium; (U) Military medicine; (U) Pneumococcus;

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Study the subcellular effects of microbial toxins and other informational molecules and determine the role of lysosomal enzymes in the catabolism of exogenous proteins.

24 (U) A variety of techniques, e.g. fractionation, electron microscopy are used to study subcellular action of toxins.

25 (U) 70 07 - 71 06 - Staphylococcal enterotoxin B (SEB) was found to cause a dose-dependent swelling of isolated rabbit liver mitochondria. This phenomenon was characterized and found to be nonenergy dependent, unaccompanied by membrane lipid peroxidation, and could be prevented by addition of specific antitoxin, Mg ions or EDTA. ATPase activity increased with swelling, though ATP was unable to contract SEB-swollen mitochondria. Cytochrome oxidase activity was inhibited in intact mitochondria exposed to SEB. These findings suggested SEB binding to mitochondrial membranes, with resultant structural alterations, facilitating entry of ions and water into mitochondrial membranes and compartments. The presence of SEB in rabbit liver was demonstrated by liquid scintillation measurements and autoradiography. It was hypothesized that mitochondrial swelling in selected tissues in vivo, with resultant impairment of function, could account for the clinical syndrome associated with SEB intoxication. Based on release of ninhydrin-positive material from hydrolysates and immuno- and disc gel electrophoretic characterization of hydrolysates, results indicated that this toxin is relatively resistant to hydrolytic attack by lysosomal proteases. In addition, fluorescein tag was found to be readily cleaved from SEB by lysosomal enzymes, suggesting that studies which utilize release of this label is an indicator of SEB catabolism are subject to erroneous interpretation. Study of Diplococcus pneumoniae in rats was begun.

Publications: Fed. Proc. 30:600, 1971; J. Infect. Dis. 1971, In press.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 01: Vulnerability of Man to Biological Agents

Work Unit No. 096 01 803: Subcellular Biological Effects of Microbial Toxins and Microbial Disease

Description:

Study the subcellular effects of microbial toxins and other informational molecules, and determine the role of lysosomal enzymes in the catabolism of oxogenous proteins.

Progress, Part I:

To further elucidate the mode of action of staphylococcal enterotoxin B (SEB), its effects on subcellular organelles and enzyme systems were investigated. It was found that SEB caused a dose-dependent swelling of isolated rabbit liver mitochondria. A series of experiments was designed to characterize this phenomenon, and to evaluate whether it could explain the clinical effects of SEB. Detailed descriptions and results of these experiments have been reported[1/] (J. Infect. Dis. In press).

Briefly, mitochondria isolated by differential centrifugation of rabbit liver homogenate, were incubated at room temperature with SEB for 60 min. During the incubation period, optical density measurements were recorded at 520 nm; decreasing optical density denoted mitochondrial swelling. This swelling was found to be specific for rabbit liver mitochondria; lung, gut and heart mitochondria were not affected by SEB. Kidney mitochondria were only slightly affected. Prior addition of specific equine antitoxin prevented swelling of liver mitochondria induced by SEB. Cyanide did not prevent the mitochondrial response to SEB, indicating the nonenergy-dependent nature of this phenomenon. Increases in malonyl dialdehyde levels were not observed during swelling, suggesting this response was not mediated by deterioration of mitochondrial membranes through peroxidation of membrane lipids. SEB-induced swelling was prevented by addition of 2 membrane stabilizers, Mg++ and EDTA, suggesting that the effects of SEB may be the result of direct interaction of the toxin with mitochondrial membranes. Further evidence for this hypothesis was provided by the finding that cytochrome oxidase activity was substantially inhibited in intact, but not in disrupted, mitochondria exposed to SEB. Inhibition of enzyme activity may have been due to a competition for membrane binding or transport sites between the substrate, cytochrome C, and SEB, both cationic proteins. Also, ATP was unable to produce contraction of SEB-treated mitochondria, although the ATPase activity in these mitochondria increased to twice that of controls. This finding further suggested that SEB interacted with mitochondrial membranes, facilitating hydrolysis of ATP by unmasking membrane-bound ATPase.

These findings suggest that a mechanism of action of SEB may be to cause alterations in structure of mitochondrial membranes by interacting with periodically recurring negatively charged groups in the membrane. The alterations in the membrane may facilitate the entry of ions, e.g. K+, into or through the membrane, followed by accumulation of water inside mitochondrial membranes and compartments.

SEB-induced swelling of mitochondria in vivo could account for the clinical signs and symptoms associated with SEB intoxication. Mitochondrial swelling would result in loss of ions, substrates and cofactors from within mitochondrial compartments and contribute to degeneration of mitochondrial functions.

In support of this proposed mechanism of action, the ability to demonstrate localized SEB becomes an important prerequisite. Using tritium ^{3}H SEB, it was determined by liquid scintillation measurements of rabbit tissue extracts that, in addition to other tissues, there was accumulation within the liver. This was confirmed by autoradiographic examination of rabbit liver 2 hr post-challenge with tritiated SEB, which revealed silver grains overlying hepatic parenchymal cell cytoplasm.

Summary, Part I:

SEB was found to cause a dose-dependent swelling of isolated rabbit liver mitochondria. This phenomenon was characterized and found to be nonenergy dependent, unaccompanied by membrane lipid peroxidation, and could be prevented by addition of specific antitoxin, Mg++ or EDTA. ATPase activity increased with swelling, although ATP was unable to contract SEB-swollen mitochondria. Cytochrome oxidase activity was inhibited in intact mitochondria exposed to SEB. These findings suggested SEB binding to mitochondrial membranes, with resultant structural alterations, facilitating entry of ions and water into mitochondrial membranes and compartments. The presence of SEB in rabbit liver was demonstrated by liquid scintillation measurements and confirmed by autoradiographic techniques. It was hypothesized that mitochondrial swelling in selected tissues in vivo, with resultant impairment of function, could account for the clinical syndrome associated with SEB intoxication.

Progress, Part II:

SEB has been found to accumulate within lysosomes of rabbit kidney cells[2/] (CES Annual Report, FY 1970, p. 93). Since lysosomes contain a variety of proteases capable of digesting exogenous proteins[3/] (Lysosomes in Biology and Pathology, Vol. 2, p. 216), a study was initiated to determine their role in the metabolism of SEB. Lysosomal proteases extracted from purified liver and kidney lysosomes[4, 5/] (Arch. Int. Physiol. 72:698, 1964); J. Ultrastruct. Res. 16:13, 1966) were used to digest SEB in vitro at 37 C for up to 72 hr. Digestion of toxin was determined by monitoring the increase of ninhydrin-positive material in the incubation mixture.

Initial experiments measured catabolism of SEB as a function of pH. A broad pH activity profile was found with a peak of 4.0 - 5.2. Maximum release of ninhydrin-positive material could be obtained by using a 1:20 ratio of lysosomal enzyme extract to SEB.

The amount of this material released by exaustive lysosomal digestion when compared to that released by total hydrolysis of the protein with 6N HCl indicated that approximately 8% of the molecule was hydrolyzed by lysosomal proteases. Disc gel electrophoresis and immunoelectrophoresis showed that fragmentation of the toxin did not occur. Predominant amino acids released by lysosomal digestion were lysine, threonine, tyrosine, asparagine, aspartate, threonine and isoleucine. These were found in the ratio of 10:3:3:3:2:1, respectively. Only trace amounts of proline, glycine, glutamine and serine, were observed. These findings suggest that lysosomal digestion resulted in cleavage of a small portion of the carboxyl end of the SEB molecule. Furthermore, this data suggested that lysosomal endopeptidases (Cathepsin D) are as ineffective as gastrointestinal proteases (trypsin, pepsin)[6] (Lysosomes in Biology and Pathology, Vol. 2, p. 245) in fragmenting the toxin. The observed cleavage of amino acids from the carboxyl end of an SEB molecule may be due to the action of a lysosomal carboxypeptidase (Cathepsin A).

When fluorescein-labelled SEB was digested by lysosomal enzymes, a substantial portion of the fluorescein, 48% in 36 hr, was removed from the molecule. Chromatography on Sephadex G-50 showed that the label was still attached to an amino acid moiety. The majority of the protein, however, showed an elution pattern similar to that of control SEB. These experiments suggest that evaluation of SEB catabolism, by monitoring the release of fluorescein label, may lead to erroneous interpretations since it only reflects the release of a small number of labelled amino acids from the carboxyl end of the molecule rather than an extensive digestion of the toxin.

Lysosome-treated SEB was found to react with specific antiserum. Administered orally to monkeys it demonstrated the same toxicity as native toxin.

The effects of kidney lysosomal proteases on SEB catabolism were similar to those described above using liver lysosomal hydrolases.

Summary, Part II:

Studies of in vitro catabolism of SEB by lysosomal enzymes are described. Based on release of ninhydrin-positive material from hydrolysate, and immuno- and disc gel electrophoretic characterization of hydrolysates, results indicated that this toxin is relatively resistant to hydrolytic attack by lysosomal proteases. In addition, fluorescein tag was found to be readily cleaved from SEB by lysosomal enzymes, suggesting that studies which utilize release of this label as an indicator of SEB catabolism are subject to erroneous interpretation.

Progress, Part III:

Because resistance of SEB to lysosomal hydrolysis was an unexpected finding, additional studies were performed to determine the catabolism of various other proteins by lysosomal hydrolases (Table I). These proteins were incubated in a solution containing 5% lysosomal proteins, prepared as described in Part II, for 72 hr at 37 C, pH 4.4. Hemoglobin, the usual substrate for cathepsin D, was 59% hydrolyzed and bovine serum albumin, 39%, following this treatment. ACTH, insulin, casein, fibrinogen, were also significantly digested. γ globulin, SEB, chymotrypsinogen, protamine, gelatin, RNase and lysozyme were found to be relatively resistant to lysosomal digestion. The latter proteins, which exhibited the greatest resistance to hydrolysis, were, with the apparent exception of gelatin, cationic proteins. Proteins that were readily hydrolyzed, on the other hand, were primarily anionic proteins.

TABLE I. EXHAUSTIVE DIGESTION OF PROTEIN BY LYSOSOMAL EXTRACTS

PROTEIN	% HYDROLYZED IN 72 HR Mean ± SEM	ISOELECTRIC POINT
Hemoglobin	59.0 ± 9.2	6.9
BSA	38.8 ± 4.6	4.7 - 4.9
ACTH	32.3	4.6
Fibrinogen	23.5 ± 3.6	5.5 - 5.8
Cytochrome C	19.7 ± 6.4	9.8 - 10.0
Casein	17.9	4.0
Insulin	17.6 ± 0.0	5.2
Chymotrypsinogen	8.8	9.5
7S γ Globulin	8.2 ± 1.6	7.3 - 8.2
Gelatin	7.0 ± 3.0	4.7 - 5.0
SEB	6.9 ± 1.0	8.6
Lysozyme	4.9 ± 1.0	11.1
RNAase	4.4 ± 1.5	9.6
Protamine	0.8	12.1

Protein hydrolysates were further characterized by basic and acidic disc-gel electrophoresis. Degradation of hemoglobin was so complete that no protein bands were observed following lysosomal digestion. Other proteins such as BSA, γ globulin, fibrinogen and gelatin were fragmented into a number of smaller polypeptides. Electrophoretic patterns indicated that the majority of resulting fragments were positively charged residues. In contrast, electrophoretic patterns of cationic proteins following 72 hr of lysosomal digestion were similar to undigested controls indicating that fragmentation or degradation had not occurred.

Lysosomal-treated γ globulin and fibrinogen were tested for immunogenicity. Serological responses of rabbits immunized with 50 μg of digested antigen are shown in Table II. Though higher hemagglutination (HA) titers were obtained after vaccination with native proteins, lysosomal-treated fibrinogen or γ globulin produced substantial HA titers. Agar gel precipitin analysis demonstrated that antibodies produced by digests of fibrinogen and γ globulin identified with those produced by the corresponding control antigens. These data indicate that hydrolysis of these 2 proteins by lysosomal enzymes releases fragments that are still immunogenic, and that, since immunological specificity is usually directed at the tertiary structure of antigens, the fragments are structually intact. The data also support the hypothesis that the lysosomes of phagocytic cells may participate in immune responses by phagocytizing and digesting particulate and soluble antigens, thereby exposing immunogenic sites, then releasing nondegradable cationic fragments to the immune system as specific immunogenic moieties.

TABLE II. SEROLOGICAL RESPONSE OF RABBITS ADMINISTERED LYSOSOMAL DIGESTED AND NATIVE ANTIGEN

IMMUNIZING ANTIGEN	TREATMENT	REACTION WITH NATIVE ANTIGEN	
		HA Titer	Precipitin[a/]
Fibrinogen	None	640	+
	Lysosomal hydrolysis 72 Hr	80-320	+
7S γ Globulin	None	1280-2560	+
	Lysosomal hydrolysis	160-320	+

a. Identifies with reaction of control sera.

Summary, Part III:

Studies of *in vitro* catabolism of various proteins by lysosomal hydrolases have been conducted. Results indicated that cationic proteins showed greatest resistance to lysosomal hydrolysis, as measured by release of ninhydrin-positive material, disc-gel electrophoretic and immunoelectrophoretic characterization of hydrolysates. In addition, the serological responses to hydrolysates of fibrinogen and γ globulin suggested that immunogenic fragments were released after lysosomal hydrolysis. The importance of these findings in relation to antigen handling by macrophages is mentioned.

Progress, Part IV:

Enterotoxemia experimentally produced in rhesus monkeys by continuous infusion of SEB, resulted in characteristic dose-related electron microscopic lesions. The pulmonary endothelium exhibited focal disruption of the cell and selective swelling of the cytoplasmic contents. Changes in subcellular organelles were not noted; basement membranes, likewise, were unaffected regardless of the absence of both pneumocytes and endothelial cell layers. No endothelial cell emboli were seen. Results suggest a close relationship between leukocytes, sequestration and degeneration, and endothelial change damage. A manuscript has been written for submission to a journal.

Summary, Part IV:

Ultramicroscopic studies were conducted on tissues of monkeys continuously infused with SEB.

Progress and Summary, Part V:

A new study was begun to evaluate effects of an infection upon function and integrity of liver lysosomes, mitochrondria, and perorisomes. In the first model rats will be infected with *Diplococcus pneumoniae*. Livers will be assayed for proteins and a series of enzymes using density gradient zonal centrifugation.

Presentation:

1. Canonico, P. G. Lysosomal processing of exogenous proteins. Presented at Annual Meeting of Federation of American Societies for Experimental Biology, Chicago, Ill. 12-17 April 1971.

Publications:

1. Canonico, P. G., M. J. Van Zwieten, and R. S. Pinkerton. 1971. Lysosomal processing of exogenous proteins. Fed. Proc. 30:600 (abstract).

2. Canonico, P. G., and M. J. Van Zwieten. 1971. Swelling of rabbit liver mitochondria induced by staphylococcal enterotoxin B. J. Infect. Dis. In press.

LITERATURE CITED

1. Canonico, P. G., and M. J. Van Zwieten. 11971. Swelling of rabbit liver mitochondria induced by staphylococcal enterotoxin B. J. Infect. Dis. In press.

2. Commission on Epidemiological Survey. Dec. 1970. Armed Forces Annual Report FY 1970 to the Epidemiological Board. p. 93 to 109. Fort Detrick, Maryland.

3. Tappel, A. L. 1969. Lysosomal enzymes and other components, p. 216 to 240. In J. T. Dingle and H. B. Fell (eds.), Lysosomes in Biology and Pathology. Vol. 2. American Elsevier Publ. Co., New York.

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5. Maunsbach, A. B. 1966. Isolation and purification of acid phosphatase-containing autofluorescent grannules from homogenates of rat kidney cortex. J. Ultrastruct. Res. 16:13-34.

6. Barrett, A. J. 1969. Properties of lysosomal enzymes, p. 245 to 312. In J. T. Dingle and H. B. Fell (eds.), Lysosomes in Biology and Pathology. Vol. 2. American Elsevier Publ. Co., New York.

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ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 02:	Prevention and Treatment of Biological Agent Casualties
Work Unit No. 096 02 002:	Evaluation of Efficacy of Experimental Vaccines in Man.
Reporting Installation:	U.S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Divisions:	Medical and Microbiology
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Authors:	Peter J. Bartelloni, Lt Colonel, MC (I-V) Nemesio M. Francisco, Major, MC (I-V) Venton R. Harrison, WRAIR (II) Burton A. Dudding, Major, MC, WRAIR (IV) Philip K. Russell, Lt Colonel, MC (IV) John D. Marshall, Jr., Colonel, MSC (III)
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
	DA OL0829	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 01 06	D. CHANGE	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO. CODES*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	02	002
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	CDOG 1212b(9)			

11. TITLE (Precede with Security Classification Code)*
(U) Evaluation of Experimental Vaccines in Man in BW Defense Research (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*
003500 Clinical Medicine; 004900 Defense; 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
61 10	CONT	DA	C. In-house

17. CONTRACT GRANT
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER*:
c. TYPE: NA
d. AMOUNT:
e. KIND OF AWARD:
f. CUM. AMT.:

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	2	190
	CURRENT 72	2	160

19. RESPONSIBLE DOD ORGANIZATION
NAME:* USA Medical Research Institute of Infectious Diseases
ADDRESS:* Fort Detrick, Md 21701

RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
NAME:* Medical Division USAMRIID
ADDRESS:* Fort Detrick, Md 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME:* Bartelloni, P. J.
TELEPHONE: 301 663-4111 Ext 6135
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Francisco, N. M.
NAME: DA

21. GENERAL USE
Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Vaccines; (U) Immunization; (U) Encephalitis, equine (EEE); (U) Military Medicine; (U) Chikungunya; (U) Adenovirus Type 21; (U) Plague; (U) Human volunteer, Rift Valley Fever

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Evaluate experimental vaccines developed by various contractors, organizations or other governmental agencies.

24 (U) Test vaccines are given to experimental animals, and when considered safe, to volunteers.

25 (U) 70 07 - 71 06 - Inactivated Rift Valley Fever Vaccine was evaluated in man by administration of 2 doses of 0.5 ml each 28 days apart; neutralizing antibody were adequate in majority of subjects up to day 270 but not on day 360.

Chikungunya Vaccine, Inactivated, Lot E-20, was evaluated in a year long study in volunteers. The vaccine was found to be of low reactogenicity and high antigenicity when administered in 2 doses of 0.5 ml or 1.0 ml 28 days apart.

Plague Vaccine, USP (E Medium) was evaluated in volunteers. Each subject was administered a primary dose of 1.0 ml and booster doses of 0.2 ml on days 90 and 270. A few mild local reactions occurred. Primary immunizations resulted in hemagglutination (HA) antibody responses in 83% of subjects. Subsequent administration of booster doses of vaccine resulted in HA response in 93% of subjects on day 270.

Adenovirus Vaccine, Live, Oral, Type 21, was found to be of low reactogenicity, and to elicit a satisfactory neutralizing antibody response in 90% of volunteers 28 days after administration of vaccine. No evidence of communicability amoung unvaccinated controls was found.

An inactivated EEE Vaccine produced by large scale production methods was given to a number of volunteers in 2 different dosages 28 days apart. No significant clinical reactions occurred. Serological results are not yet available.

Publications: J. Immun. 1971. In press. J. Infect. Dis. 1971. In press.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 096 02 002: Evaluation of Efficacy of Experimental Vaccines in Man.

Description:

Evaluation of experimental vaccines developed by various organizations, contractors, or other governmental agencies.

Progress, Part I:

Evaluation of Inactivated Rift Valley Fever Vaccine (Medical Division Project No. FY 69-10): A year long study in 16 volunteers to determine the clinical and serological responses to 2 doses of 0.5 ml each of Rift Valley Fever (RVF) Vaccine, Inactivated, Tissue Culture Origin, NDBR 103, Lot 6 (National Drug Co.) was completed. Clinical, laboratory results and serological determinations on specimens obtained up to day 90 were reported previously[1] (USAMRIID Annual Progress Report FY 1970, p. 105). Serum neutralization tests performed on blood specimens from vaccinees for the entire study are shown in Table I.

As shown, the majority of subjects did not achieve significant neutralizing antibody until 14 days after the administration of the second dose of vaccine. Mean LNI remained significant thereafter until day 360 when the mean LNI decreased to 1.6 logs. As shown in Table I, the majority of subjects maintained significant antibody from days 42 through 270. On day 360 only 6 of 14 subjects (43%) had adequate neutralizing antibody: of these, 5 showed a decrease in neutralizing antibody titer below 1.7 logs between days 270 and 360, one volunteer achieved and maintained significant antibody from day 14 to day 180 only, one subject failed to develop adequate neutralizing antibody following vaccination, an additional subject failed to maintain significant antibody after day 56.

Summary, Part I:

Serological responses were evaluated on blood specimens obtained from 16 volunteers administered 2 doses of 0.5 ml each of RVF vaccine 28 days apart. The majority of subjects developed adequate neutralizing antibody by 14 days after the second dose of vaccine, and mean LNI were significant

TABLE I. NEUTRALIZING ANTIBODY REPONSES[a] IN 16 VOLUNTEERS WITHOUT PRIOR RVF EXPERIENCE TO THE ADMINISTRATION OF 2 DOSES OF 0.5 ML EACH OF RVF VACCINE 28 DAYS APART.

VOLUNTEERS	LOG_{10} SNI BY DAY AFTER THE FIRST DOSE							
	14	28	42	56	90	180	270	360
SBA	1.9	1.9	3.0	2.8	2.5	2.1	1.5	1.5
FLL	2.0	1.4	2.5	2.5	2.1	2.7	2.5	1.6
RWR	1.5	1.3	2.3	2.2	2.0	3.2	2.9	2.6
SLB	0.7	0.6	2.4	2.4	2.6	2.4	2.3	1.0
KWJ	0.0	2.3	1.4	1.4	1.8	2.7	2.0	0.0
VVE	0.4	0.9	1.7	1.0	1.1	0.7	0.0	0.6
CRR	1.2	1.9	2.1	ND	1.1	3.1	2.1	ND
JLS	0.8	1.2	1.5	2.3	1.2	2.0	2.5	1.5
GLS	1.4	1.9	2.5	2.0	2.0	3.0	1.7	2.3
EJR	2.1	1.4	2.1	2.3	1.6	2.0	1.7	1.0
JAR	2.2	2.4	2.0	2.2	1.6	2.5	3.0	2.5
MVM	1.1	1.0	2.0	2.0	1.5	1.0	0.5	1.3
RBH	1.0	0.9	0.5	0.9	0.4	2.9	2.1	2.6
DRS	1.7	1.8	2.4	2.5	2.5	2.2	2.2	2.2
DES	0.9	1.4	1.5	1.8	2.4	ND	1.8	1.8
REE	2.6	2.1	ND	2.0	2.5	ND	1.3	ND
MEAN	1.3	1.5	2.0	2.0	1.8	2.3	1.9	1.6
No. $\geq$ 1.7 Logs	6/16	7/16	11/15	12/15	9/16	12/14	12/16	6/14
%	38	44	73	80	56	86	75	43
RANGE	(0.0-2.6)	(0.6-2.4)	(0.5-3.0)	(0.9-2.8)	(0.4-2.6)	(0.7-3.2)	(0.0-3.0)	(0.0-2.6)

a. No subject had demonstrable neutralizing antibody on Day 0.
b. ND = Not done.

until day 360. The majority of vaccinees had significant neutralizing antibody at various times after vaccination until day 360 when only 6 of 14 subjects or 43% had significant neutralizing antibody.

Progress, Part II:

Evaluation of Inactivated Chikungunya Vaccine, Dried, Tissue Culture Origin, Lot E-20 (Medical Division Project No. FY 70-5): Clinical and laboratory results in 16 volunteers administered two 0.5 ml or 1 ml doses of Chikungunya vaccine 28 days apart were reported previously.[1] Blood was obtained for serological tests prior to vaccination and on days 14, 28, 42, 56, 90, 180, 270, and 360. Tissue culture serum neutralization tests, complement-fixation (CF) and hemagglutination-inhibiting antibody (HI) determinations were performed by Mr. V. R. Harrison, Department of Virus Diseases, Division of Communicable Disease and Immunology, Walter Reed Army Institute of Research, Washington, D. C. Table II shows these serum neutralization indices. Although not shown in the table, the majority of subjects in both Groups I and II achieved significant neutralizing antibody 14 days following the first dose of vaccine. However, not until 14 days following the second dose of vaccine, day 42, did all subjects develop significant neutralizing antibody with a mean LNI of 2.7 logs in both Groups I and II.

TABLE II. SEROLOGICAL RESPONSES OF VOLUNTEERS WITHOUT PRIOR CHIKUNGUNYA EXPERIENCE TO THE ADMINISTRATION OF 2 DOSES OF CHIKUNGUNYA VACCINE.

DAY	MEAN LNI* BY DAY (Range) Group I (0.5+0.5 ml)		MEAN LNI* BY DAY (Range) Group II (1.0+1.0 ml)	
0	0		0	
14	1.8	(0.7-2.7)	1.9	(1.3-2.5)
28	1.9	(1.3-3.0)	1.9	(1.0-3.0)
42	2.7	(2.0-3.5)	2.7	(2.0-3.5)
56	2.6	(1.4-3.4)	2.8	(2.4-3.3)
90	2.6	(1.7-3.6)	2.9	(2.0-4.0)
180	2.5	(1.7-3.0)	2.4	(1.4-3.6)
270	2.4	(1.7-3.0)	2.5	(1.4-3.6)
360	2.2	(0.7-3.6)	2.7	(1.4-3.6)

* Log_{10} serum neutralization test.

Mean LNI remained significant and neutralizing antibody persisted to day 360 in both groups. Although the mean LNI on days 56 through 360 were slightly higher in Group II, they were not considered to be significantly different. Only one subject in Group I had an LNI < 1.7 logs on day 360; although this volunteer had achieved an LNI of 2.3 logs on day 42, his titer had decreased to 1.4 logs on day 56, but increased to 1.7 logs on day 90 and remained at this level until day 360 when it decreased to 0.7 logs. Only one subject in Group II had an LNI < 1.7 logs on day 360. This volunteer had a significant neutralizing antibody titer on day 42 which persisted to day 90 but decreased on day 180 to 1.4 logs and remained at this level through day 360. CF and HI tests remained low throughout the entire study and did not correlate with neutralizing antibody titers. There did not appear to be any significant differences in mean neutralizing antibody titers obtained in either Group I or II.

<u>Summary, Part II</u>:

Chikungunya Vaccine, Inactivated, Lot E-20, was evaluated in volunteers. The vaccine was found to be of low reactogenicity and of high antigenicity when administered in 2 doses of 0.5 ml or 1.0 ml 28 days apart.

<u>Progress, Part III</u>:

<u>Evaluation of the clinical and serological responses of volunteers to the administration of Plague Vaccine USP (E Medium) (Medical Division Project No. FY 70-7)</u>: Twenty-nine healthy male volunteers were administered 1.0 ml of Plague Vaccine, USP (E Medium) intramuscularly in the deltoid region of the arm on day 0 and a 0.2 ml booster on day 90 and 270 in an identical manner. Each vaccinee was observed 24 and 48 hr following each vaccination to determine reactogenicity. Blood was obtained from each vaccinee prior to vaccination and on days 15, 30, 60, 90, 105, 120, 150, 180, 210, 240, 270, 285, 300, and 330 for hemagglutination (HA) and complement fixation (CF) tests. Additional blood specimens will be obtained on days 360 and 390.

After the initial dose, 26 volunteers experienced local soreness. In 2 of these, erythema ranging from 2-5 mm in diameter developed, in one man the erythema was associated with pruritus. One vaccinee complained of feverish sensation and 3 other participants remained asymptomatic.

Following the booster dose, 15 volunteers experienced local soreness, in addition, 5 had erythema ranging from 2-20 mm in diameter, 3 had induration of 2-3 mm in size, and 3 had associated axillary adenopathy. One volunteer developed headache and had an oral temperature of 99.4 F within 8 hr of the injection.

Following the second booster dose of vaccine, 11 subjects had no local or systemic reactions. Eighteen subjects developed soreness at the injection site which was mild in severity and did not interfere with performance of duty.

In only 4 subject did local symptoms persist for 72 hr. In the remainder symptoms persisted for · 24 - 48 hr. Two individuals developed erythema of 1 cm or less 24 hr following inoculation which disappeared by the 3rd day following vaccination.

Table III shows the serological responses for the first 11 months. Twenty-four of 29 subjects developed HA antibodies in response to the initial immunization. The geometric mean titer (GMT) was 1:25. By day 90, the GMT had declined to 1:19. All 29 individuals failed to produce CF antibodies following the first immunization.

Twenty-six individuals showed a booster response as measured by HA antibody following the second inoculation. The GMT on day 105 was 1:140. During the following 6 mon period there was a gradual decline in HA antibody in all individuals. The GMT at day 270 was 1:24. CF antibodies were detected in 19 individuals on day 105. However, by day 180 the CF titer became negative in all but 1 subject.

The second booster inoculation stimulated HA antibody production in 27 individuals resulting in a GMT of 1:576 on day 285 which was followed by a gradual decline in titers during the next 2 mon. The CF antibody response to the second booster was essentially the same as seen previously.

It is interesting to note that 5 individuals failed to respond to the primary immunization and that subsequent booster inoculations reduced this number to 2.

<u>Summary, Part III</u>:

Twenty-nine individuals administered a primary and 2 booster inoculations of Plague Vaccine experienced mild local reactions. Primary immunization resulted in HA antibody response in 83%. The subsequent administration of booster doses of vaccine on days 90 and 270 resulted in production of measurable HA antibody in 90 and 93% of the individuals respectively.

<u>Progress, Part IV</u>:

<u>Evaluation of Adenovirus Vaccine, Live, Oral Type 21, Lot 16C1X-01201 (L-AV-21) (Medical Division Project No. FY 71-2)</u>: The clinical, serological safety, infectivity, and communicability of L-AV-21 were evaluated in volunteers.

The vaccine virus used for immunization was an adenovirus Type 21 strain (V-270) obtained by Wyeth Laboratories from Dr. R. Chanock, National Institutes of Allergy and Infectious Diseases, National Institutes of Health. The strain was isolated in human embryonic kidney (HEK) cell cultures, passaged 3 additional times in HEK cultures and 11 times in human diploid fibroblast (WI-38) cultures. The product was lyophilized, mixed with an inert vehicle and

TABLE III. HEMAGGLUTINATION AND COMPLEMENT-FIXING ANTIBODY RESPONSES OF 29 INDIVIDUALS ADMINISTERED PLAGUE VACCINE USP (E MEDIUM)

VACCINE DOSE		HA POSITIVE			CF POSITIVE		
ML	DAY	No.	%	GMT[a]	No.	%	GMT[a]
	0	2	7	0.2	0		
1.0	0						
	15	24	83	25	0		
	30	25	86	22	0		
	60	25	86	27	0		
	90	24	83	19	0		
0.2	90						
	105	26	90	140	19	66	6.0
	120	26	90	106	16	55	4.0
	150	26	90	96	7	5	1.5
	180	25	86	67	3	10	0.7
	210	24	83	48	2	6	0.5
	240	24	83	44	1	3	0.2
	270	23	79	24	1	3	0.2
0.2	270						
	285	27	93	576	17	59	6.5
	300	27	93	383	17	59	5.2

a. Reciprocal.

prepared into enteric coated capsules (Lot 16CIX-01201) by Wyeth Laboratories. The capsules contained $10^{6.4}$ median tissue culture infectious doses ($TCID_{50}$) as titrated in HEK cell cultures.

Fifteen healthy male volunteers found to be free of demonstrable adenovirus Type 21 antibody by tissue culture neutralization tests were studied. Ten subjects were fed $10^{6.4}$ $TCID_{50}$ of L-AV-21. Five volunteers were administered enteric coated placebo capsules. Volunteers were housed on two closed research wards; each ward contained volunteers who received vaccine and subjects who received placebo. All subjects were examined at least twice daily for 21 days following exposure for evidence of respiratory disease or other untoward reaction. Each subject had frequent laboratory determinations for white blood cell and differential counts, hematocrit, platelet counts, serum glutamic oxalacetic transaminase, serum glutamic pyruvic transaminase, direct and indirect bulirubin, blood urea nitrogen and alkaline phosphatase determinations. The above tests were performed according to standardized laboratory procedures.

Blood was obtained at 0800 hr from each subject on days -4, 0, 7, 10, 14, 18, 21, and 28 for serological studies. Serum neutralization tests were performed on serum samples from volunteers in HEK tube cultures using Adenovirus Type 21 Vaccine Strain (V-270). Serum neutralization end-points were determined at a time when the test dose of virus showed 10 $TCID_{50}$ in HEK tube cultures. Adenovirus CF titers on blood obtained on days 0 and 28 were determined by a standard microtiter procedure against a commercial adenovirus CF antigen obtained from Microbiological Associates. Throat washings and stool (or rectal swab) specimens were obtained on each volunteer on study days -4, -3, -2, -1, 0, and daily through day 28; 0.3 ml aliquots of a 10% suspension of each stool specimen were inoculated into 2 HEK tube cultures. Tubes were incubated at 36 C and observed for cytopathic effect (CPE) every other day. Isolates exhibiting characteristic CPE were typed in tissue culture neutralization tests in HEK tube cultures with hyperimmune ADV Type 21 antiserum.

No meaningful changes in clinical laboratory values were found in the vaccinees or the placebo group of subjects.

ADV-21 nasopharyngeal excretion was not demonstrated in throat washings of either vaccinees or those subjects administered a placebo.

The pattern of ADV-21 excretion in the stool is shown in Table IV. Nine of 10 volunteers receiving L-AV-21 excreted adenovirus Type 21 in the stool. ADV-21 shedding was demonstrated first on day 4 and last on day 21 of the study. Duration of fecal shedding varied between 4 days and 17 days. None of the 5 volunteers receiving the placebo tablet excreted ADV-21 in the stool during the study. One immunized volunteer, No. 3, excreted ECHO virus Type 6 in his stool from day -4 to day 12 of the study.

TABLE IV. ADENOVIRUS EXCRETION IN STOOL IN SUBJECTS RECEIVING L-AV-21*

VOLUNTEER NUMBER	ONSET OF RECTAL SHEDDING day	DURATION OF EXCRETION days
1	6	8
2	6	11
3	6	10
5	14	4
6	10	11
8	7	10
10	6	11
11	4	17
13	5	15
15	0	0

* No adenovirus Type 21 excretion was found in those subjects administered a placebo.

Table V shows the ADV-21 neutralization titers of volunteers prior to the administration of vaccine and a placebo on day 28 of the study. Neutralizing antibody was not detected in the 28-day serum of the 5 placebo volunteers or in one immunized volunteer who failed to excrete ADV-21.

Complement-fixation tests were negative before vaccine administration and on day 35 in both vaccinees and controls.

Antibody responses of the 9 immunized ADV-21 excretors were variable; two showed detectable neutralizing antibody only at a 1:2 serum dilution, whereas 2 others had titers of 1:64. Repeat neutralization tests using a wild ADV-21 isolated from a subject at Fort Dix, New Jersey in 1967 gave similar results.

Mild, afebrile upper respiratory disease or diarrhea was noted in 3 immunized and 2 control volunteers. Relationship of these illnesses to ADV-21 stool excretion are summarized in Table VI.

TABLE V. ADV-21 NEUTRALIZING ANTIBODY TITERS IN SUBJECTS ADMINISTERED ORAL L-AV-21 AND AN ENTERIC COATED PLACEBO CAPSULE.

	VOLUNTEER NUMBER	DAY OF EXCRETION	NEUTRALIZATION TITERS[a/] RECIPROCAL 28 Day
IMMUNIZED			
Excretors ADV-21	1	8	2
	2	11	32
	3	10	64
	5	4	4
	6	11	8
	8	10	2
	10	13	32
	11	17	64
	13	15	64
Non-Excretor	15	0	<2
PLACEBO	4	0	<2
	7	0	<2
	9	0	<2
	12	0	<2
	14	0	<2

a. Day 0 titers were < 1:2

TABLE VI. RELATIONSHIP BETWEEN ADV-21 RECTAL SHEDDINC AND ILLNESS

VOLUNTEER NUMBER	STOOL EXCRETION ADV-21 (Study Days)	RESPIRATORY DISEASE (Study Days)	DIARRHEA (Study Days)
IMMUNIZED			
15	None	7	9 · 16
10	6,8 - 16,18,20	13 - 14	None
1	6,8 - 13,15	None	16 - 18
PLACEBO			
4	None	-4 - 0	16
12	None	None	20

Volunteer No. 15 who failed to excrete ADV-21 in his stool had intermittent diarrhea from study day 9 to 16. Diarrhea in 3 other volunteers was observed between days 16 and 20. No viral pathogens or Adenovirus Type 21 were isolated from the stools collected during these periods.

Nine of 10 volunteers receiving L-AV-21 excreted ADV-21 in the stool and all excretors developed ADV-21 neutralizing antibody. The infection rate obtained with this lot of vaccine virus ($10^{6.4}$ $TCID_{50}$/capsule) appears to approach 90% and thus is entirely suitable for use in man.

ADV-21 was not recovered from the pharynx of immunized volunteers. No evidence of communicability of the vaccine virus was found in 5 controls given a placebo, who also lacked detectable serum ADV-21 neutralizing antibody and who were housed together with the immunized group.

In all immunized volunteers who excreted ADV-21, vaccine neutralizing antibody was present in the 28-day serum.

Summary, Part IV:

Adenovirus Vaccine, Live, Oral, Type 21 was administered to 10 volunteers. It was found to be safe, of low reactogenicity and high antigenicity. Nine of 10 vaccinees excreted ADV-21 in the stools for a

variable period. No pharyngeal excretion of ADV-21 was observed. No evidence of communicability of vaccine virus was obtained. Ninety percent of vaccinees developed infection when administered an oral dose of approximately $10^{6.4}TCID_{50}$ of ADV-21 vaccine.

Progress, Part V:

Evaluation of Eastern Equine Encephalitis (EEE) Vaccine, Formalin-Inactivated, Tissue Culture Origin, NDBR 104 (Medical Division Project No. FY 71-4 and 71-5): Clinical and serological evaluation of a laboratory lot of EEE vaccine, formalin-inactivated, tissue culture origin, Lot 1-1966 produced by the Virology Division, USAMRIID has been described in previous reports[2/] (USAMRIID Annual Progress Report, FY 1968, p. 89). The National Drug Company, Swiftwater, Pa. has developed methods for large scale production of this vaccine under contract to the United States Army Medical Research and Development Command (Contract No. DA49-193-MD-2125). One experimental lot has been produced for human use, this lot was divided into 2 portions designated Run 1 and Run 2 which was subjected to identical processing. The vaccine was approved for clinical trials by the Army Investigational Drug Review Board on 7 December 1970.

The initial study (Project No. FY 71-4) was for acceptability, to determine the extent, if any, of local and systemic reactions resulting from the parenteral administration of this vaccine. Four non-Whitecoat volunteers were utilized in this study. Two subjects were administered 0.1 ml of vaccine intradermally and 2 were administered 0.5 ml of vaccine subcutaneously. Each individual was observed at 30 min and at approximately 24 and 48 hr following the administration of vaccine. None of the subjects administered vaccine developed local or systemic reactions and none had a febrile response.

The second study (Project No. FY 71-5) was initiated to determine the clinical and serological responses in healthy male volunteers to the administration of 2 different doses of the vaccine. Sixteen volunteers participating in Project Whitecoat were utilized in this study. They were divided into 2 groups of 8 each and designated Groups I and II. Group I was administered 2 doses of 0.5 ml each of EEE vaccine 28 days apart. Group II was administered 0.25 ml of EEE vaccine 28 days apart. Subjects were observed 24 and 48 hr after each dose of vaccine for local and systemic reactions. Blood was obtained prior to vaccination and on days 7, 14, 28, and 35 for Eastern virus neutralizing antibody. Additional specimens will be obtained on days 42, 56, 90, 180 270, 360, and 390.

No significant local or systemic reactions developed in vaccinees following the administration of the 2 doses of vaccine. No subject developed a febrile response. Serological data on specimens are not yet available.

Summary, Part V:

An EEE vaccine produced by The National Drug Company, Swiftwater, Pa. utilizing large scale production methods has been administered to a limited number of subjects. It has been found to be of low reactogenicity when administered subcutaneously in 2 different doses 28 days apart. Serological results are not yet available.

Publications:

1. Calia, F. M. and P. J. Bartelloni. 1970. Rocky Mountain spotted fever. Laboratory infection in a vaccinated individual. JAMA 211:2012-2014.

2. Harrison, V. R., K. E. Eckels, P. J. Bartelloni, and C. Hampton. 1971. The production and evaluation of a formalin-killed Chikungunya vaccine. J. Immun. In press.

3. Top, Jr., F. H., R. A. Grossman, P. J. Bartelloni, H. E. Segal, B. A. Dudding, P. K. Russell, and E. D. Buescher. Immunization by selective intestinal infection with adenovirus type 7. J. Infect. Dis. In press.

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1. U. S. Army Medical Research Institute of Infectious Diseases. 1 July 1970. Annual progress report, FY 1970. p. 105 to 116. Fort Detrick, Maryland.

2. U. S. Army Medical Research Institute of Infectious Diseases. 1 July 1968. Annual progress report, FY 1968. p. 89 to 103. Fort Detrick, Maryland.

ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 02:	Prevention and Treatment of Biological Agent Casualties
Work Unit No. 096 02 003:	Chemoprophylaxis and Therapy of Infectious Diseases of Potential Biological Warfare Significance
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Division:	Medical
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Authors:	Peter J. Bartelloni, Lt Colonel, MC Nemesio M. Francisco, Major, MC
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
	DA OL0830	71 07 01	DD-DR&E AR 636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 01 06	D. CHANGE	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	02	003
b. CONTRIBUTING				
c. ~~CARDS 114f~~	CDOG 1212b(9)			

11. TITLE: (U) Chemoprophylaxis and Therapy of Infectious Diseases of Potential Biological Warfare Significance (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*
003500 Clinical Medicine; 004900 Defense; 003200 BW, CW, RW

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
62 09	CONT	DA	C. In-house

17. CONTRACT/GRANT
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER*:
c. TYPE: NA
d. AMOUNT:
e. KIND OF AWARD:
f. CUM. AMT.:

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	0	0
	CURRENT 72	1	15

19. RESPONSIBLE DOD ORGANIZATION
NAME*: USA Medical Research Institute of Infectious Diseases
ADDRESS*: Fort Detrick, Md 21701
RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
NAME*: Medical Division USAMRIID
ADDRESS*: Fort Detrick, Md 21701
PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME*: Bartelloni, P. J.
TELEPHONE: 301 663-4111 Ext 6135
SOCIAL SECURITY ACCOUNT NUMBER
ASSOCIATE INVESTIGATORS
NAME: Francisco, N. M.
NAME:
DA

21. GENERAL USE
Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Prophylaxis; (U) Therapy; (U) Infectious diseases; (U) Human volunteer (U) Military Medicine;

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Assess the effect of microbials and various drug regimens in various infectious diseases.

24 (U) Various drugs are tested in volunteers under strict protocol conditions.

25 (U) 70 07 - 71 06 - During the year, no tests were performed. This is a work unit needed for future work as required.

Publication: Clin. Med. 77:26-29, 1970.

*Available to contractors upon originator's approval

DD FORM 1498, 1 MAR 68 — PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 096 02 003: Chemoprophylaxis and Therapy of Infectious Diseases of Potential Biological Warfare Significance

Description:

Assess the effect of antimicrobials and various drug regimens in various diseases.

Progress and Summary:

During the year, no tests were performed.

Publications:

1. Burghen, G. A., W. R. Beisel, and P. J. Bartelloni. 1970. Influences of chloramphenicol administration on whole blood amino acids in man. Clin. Med. 77:26-29.

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ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 02:	Prevention and Treatment of Biological Agent Casualties
Work Unit No. 096 02 004:	Studies in Combined Antigens
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Division:	Bacteriology and Animal Assessment
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Authors:	William A. Christmas, Major, MC (I) Harry G. Dangerfield, Lt. Colonel, MC (I) Richard O. Spertzel, Lt. Colonel, VC (II) Frank E. Chapple, Captain, VC (II)
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION: DA OL0831	2. DATE OF SUMMARY: 71 07 01	REPORT CONTROL SYMBOL: DD DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 01 06	D. CHANGE	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	02	004
b. CONTRIBUTING				
c. ~~[illegible]~~	CDOG 1212b(9)			

11. TITLE: (U) Studies in Combined Antigens for Use in Military Medicine (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical Medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
63 04	CONT	DA	C. In-house

17. CONTRACT/GRANT: a. DATES/EFFECTIVE: EXPIRATION: b. NUMBER: c. TYPE: NA d. AMOUNT: e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	2	80
	CURRENT 72	2	79

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, Md 21701
RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION

NAME: Bacteriology and Animal Assessment Divisions
ADDRESS: USAMRIID Fort Detrick, Md 21701
PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Christmas, W. A.
TELEPHONE: 301 663-4111 Ext 7341
SOCIAL SECURITY ACCOUNT NUMBER
ASSOCIATE INVESTIGATORS
NAME: Dangerfield, H. G.
NAME: Spertzel, R. O. DA

21. GENERAL USE: Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Encephalitis, equine (VEE, EEE, WEE); (U) Rift Valley fever; (U) Chikungunya; (U) Q fever; (U) Yellow fever; (U) Military medicine; (U) Vaccines; (U) Immunization

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Determine the feasibility of combining various immunizing antigens and establish the compatibility, optimal dose, best schedule for administration, and efficacy of the combinations.

24 (U) Various antigens will be mixed in a variety of proportions and given to experimental animals for challenge studies. Promising combinations will be tested further and eventually may be tested in volunteers.

25 (U) 70 07 - 71 06 - I: Protective efficacy of an experimental pentavalent vaccine composed of WEE, EEE, RVF, CHIK and Q fever vaccines has been evaluated.

Each component of the combined preparation was compared to its analogue in monovalent form by a standard challenge with the virulent homologous strain. Results indicated that WEE, EEE, RVF and Q vaccines were as effective in a pentavalent vaccine as they were when given alone.

Difficulty was encountered in assaying CHIK vaccine: animals older than 6 weeks wer nonspecifically resistant to challenge and in addition, supply of vaccine was depleted. A new CHIK vaccine is currently being evaluated.

No local or systemic reactions were found when hypertonic combined vaccine was administered SC to rhesus monkeys, suggesting that acceptability of such a product may not be a serious problem.

II: Live VEE vaccine and the commercially available 17D vaccine strain of yellow fever were administered to monkeys at varying time intervals with respect to each other to assess interference suggested by earlier work. Such interference did in fact occur through day 28; the effect was lost by day 56.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 02: Prevention and Treatment of Biological Agent Causalties

Work Unit No. 096 02 004: Studies in Combined Antigens

Description:

Determine the feasibility of combining various immunizing antigens and establish the compatability, optimal dose, best schedule for administration, and efficacy of the combinations.

Progress, Part I:

Studies have been continued to evaluate the efficacy of a combined, pentavalent vaccine composed of the following inactivated particulate vaccines: Western equine encephalitis (WEE), Eastern equine encephalitis (EEE), Rift Valley fever (RVF), Chikungunya (CHIK) and Q fever (Q). The rationale and preliminary experiments relating to this project were described previously[1] (USAMRIID Annual Progress Report, FY 1970, p. 121).

The median intraperitoneal effective dose (ED_{50}) was determined for each vaccine by bioassay; an ED_{50} was defined as that quantity (ml) of vaccine injected intraperitoneally (IP) at 0 and 4 weeks which protected 50% of animals against a challenge with 100-1000 median IP lethal doses (LD_{50}), or median IP fever doses (FD_{50}) in the case of Q fever, of homologous virulent organisms 2 weeks after the last immunization. Characteristics of the mouse-adapted strain of virulent Chikungunya virus (CHIK 168) were such that injections of CHIK vaccine were given on days 0 and 7 and intracerebral (IC) challenge was on day 14. Challenge materials employed were: the California strain of WEE, Cambridge strain of EEE, Entebbe strain of RVF, CHIK 168 mouse-adapted strain, and Phase I *Coxiella burnetii*.

Lakeview (LV6:LAK) golden Syrian female hamsters, 8-10 weeks old, were used for assays of WEE, EEE and RVF vaccines. The hamster could not be employed for Q studies so that Hartley strain guinea pigs (Fort Detrick), 250-300 gm, were utilized. Male, Charles River, CD-1 strain (HaH/ICR) Swiss albino mice, 17-20 days old, were used for Chikungunya assays. Different challenge doses were employed to determine ED_{50} values for each component of the proposed combined vaccine, but studies with EEE and RVF vaccines indicated that ED_{50} values remained essentially the same for different levels of challenge (Table I).

TABLE I. MEDIAN INTRAPERITONEAL EFFECTIVE DOSE (ED_{50}) FOR COMPONENTS OF PENTAVALENT VACCINE.

VACCINE (origin)	CHALLENGE DOSE (LD_{50})	ED_{50} (ml)
WEE - Lot 6 (Virology Division, USAMRIID)	630	0.003
EEE - Lot 2-3 (NDBR)	40,000	0.011
	200	0.009
RVF - Lot 3 (NDBR)	1,000	0.038
	31,000	0.031
Q - Lot DP-7 (WRAIR)	500[a]	0.002
CHIK - Lot E-8 (WRAIR)	1,600	0.017
- Lot 1 (Virology Division, USAMRIID)	1,600	0.017

a. FD_{50}.

If 50 ED_{50} of each component antigen were incorporated in a pentavalent vaccine, the volume for 1 immunizing dose would be 3.1 ml, consisting of 0.1 ml Q, 0.15 ml WEE, 0.55 ml EEE, 0.85 ml CHIK and 1.5 ml RVF vaccine. Therefore, it was decided to employ 0.5 ml of RVF vaccine or 13 ED_{50}. Although this reduced an immunizing dose of pentavalent vaccine to 2.17 ml, this volume still was considered excessive for administration to mice and hamsters. To achieve further reduction in volume, we decided to resuspend the 4 lyophilized vaccines (EEE, WEE, RVF and Q) in fluid CHIK vaccine. This preparation contained 1 immunizing dose in only 0.85 ml. As expected, this product was hypertonic. Analyses indicated an osmolality of 736 millosomols with 2.4 gm/100 ml total protein, 358 mEq/L Na and 13.0 mEq/L K, with a pH 7.63.

Although a pentavalent preparation passed safety tests in mice and guinea pigs, as defined by USPHS Regulations, Title 42, Part 73, hypertonicity of the combination raised a question regarding acceptability of such a product in primates. It was elected to perform a pilot study in rhesus monkeys to evaluate local and systemic effects of a hypertonic

combined vaccine administered subcutaneously (SC). Isotonic and hypertonic combinations containing standard human doses of WEE, EEE and RVF vaccines, were prepared by reconstituting the components with recommended volumes, 0.5, 0.5 and 1.0 ml respectively, and ½ the recommended volumes of diluent. Analysis of the hypertonic preparation revealed an osmolality of 600 milliosmols, 2.48 gm/100 ml protein, 340 mEq/L Na, and 10.8 mEq/L K. Both preparations were injected SC in the shaved skin at the nape of the neck. One ml of hypertonic vaccine was administered to 4 monkeys, and an equivalent antigenic mass of isotonic form (2.0 ml), to another group of 4. Control animals received 1.0 or 2.0 ml of physiological saline. Rectal temperatures of all animals were obtained by constant temperature recording for 72 hr before and 48 hr after the single injection. The site of injection was observed daily for 2 weeks. There was no visual or tactile evidence of local inflammation in the 10 monkeys, and none of the animals developed recognizable systemic reactions.

Following completion of the assays for each component of the proposed pentavalent vaccine, it became possible to evaluate the effect of combination. Protective efficacy of each component in the pentavalent vaccine was compared to efficacy of the corresponding monovalent preparation. Schedules and routes of immunization and routes of challenge were identical to those employed in ED_{50} assays for individual vaccines. Antigenic mass and volume of each monovalent vaccine were equivalent to that component contained in the combined preparation. Results of this experiment are shown in Table II. It is apparent that WEE, EEE, RVF and Q vaccines, when given in a pentavalent preparation, were as effective in protecting against a standard homologous challenge as when the respective antigens were given alone. Although 2 animals immunized with pentavalent vaccine were not protected against Q fever challenge, the difference between the pentavalent and monovalent Q vaccine groups was not statistically significant (X^2 = 2.5).

Some difficulty was encountered with the assay technique for CHIK vaccine. It was discovered that age of mice at challenge was a critical factor; unimmunized animals older than 6 weeks demonstrated nonspecific as well as nonpredictable resistance when the challenge strain was administered IC. The commercial supplier of mice did not conform routinely to rigid requirements with respect to age. By the time this factor was realized, the available supply of CHIK vaccine had been depleted. Although median effective doses were determined, there was only enough CHIK vaccine for 1 comparative study of efficacy with pentavalent and monovalent preparations. Unfortunately, the problem of nonspecific resistance was encountered again in control mice challenged with CHIK 168 virus, and, therefore, assay for vaccine efficacy could not be interpreted. A new lot of vaccine has been prepared by Virology Division, USAMRIID, and potency assays are in progress.

TABLE 11. EVALUATION OF EFFICACY OF A PENTAVALENT COMBINED VACCINE FOR WEE, EEE, RVF, Q AND CHIK.

CHALLENGE ORGANISM	CHALLENGE DOSE (LD_{50})	PROTECTED/TOTAL (%) Pentavalent[a/] Vaccine	Monovalent[b/] Vaccine	Unimmunized Controls
WEE	11,130	10/10 (100%)	10/10 (100%)	0/8 (0%)
EEE	100	10/10 (100)	10/10 (100)	0/8 (0)
RVF	1,000	10/10 (100)	10/10 (100)	0/8 (0)
Q	1,160[c/]	8/10 (80)	10/10 (100)	1/10 (10)
CHIK[d/]	--	8/10 (80)	10/10 (100)	6/6 (100)

a. Combined WEE, EEE, RVF, Q and CHIK vaccines.

b. Vaccines homologous for challenge employed.

c. Represents median intraperitoneal fever doses.

d. Challenge experiments were carried out for CHIK, but an irregular death pattern invalidated the LD_{50} titration.

Serum samples were obtained from each experimental group and serological evaluation of responses to immunization is in progress.

Summary, Part I:

Protective efficacy of an experimental pentavalent vaccine composed of WEE, EEE, RVF, CHIK and Q fever vaccines has been evaluated.

Each component of the combined preparation was compared to its analogue in monovalent form by a standard challenge with the virulent homologous strain. Results indicated that WEE, EEE, RVF and Q vaccines were as effective in a pentavalent vaccine as they were when given alone.

Difficulty was encountered in assaying CHIK vaccine: animals older than 6 weeks were nonspecifically resistant to challenge and in addition, supply of vaccine was depleted. A new CHIK vaccine is currently being evaluated.

No local or systemic reactions were found when hypertonic combined vaccine was administered SC to rhesus monkeys, suggesting that acceptability of such a product may not be a serious problem.

Progress, Part II:

The serologic responses of monkeys were examined to determine if 2 live viral vaccines, 83rd tissue culture passage (TC83) of Venezuelan equine encephalomyelitis (VEE) and 17D strain of yellow fever (YF), interacted antagonistically or synergistically when administered in varying dosage schedules. A preliminary study[2] (USAMRIID Annual Report, FY 1969, p. 171) had shown interference by TC83 with development of serum neutralizing (SN) antibodies against YF (reported as an index SNI). A consistent, but insignificant difference in VEE hemagglutination-inhibition (HI) responses also had been seen.

To evaluate further this antagonistic effect, groups of 6 monkeys were examined for VEE, HI and SN antibodies and for YF SN antibodies (Table I). In this study, serology was performed on days 0, 7, 10. 14, 28 and 56 postvaccination with the respective virus vaccine. In the previous work[2] only 0-, 14- and 28-day serology was examined.

TABLE I. GROUPING OF MONKEYS IN INTERFERENCE STUDY OF 17D AND VEE (TC83) VACCINES

VEE VACCINE	TREATMENT		
$GPIPID_{50}$	Group	Description	Code For Tables
0	I	17D alone	17D
5,000	II	17D & TC83 simultaneously, same site	17D-TC83
	III	17D & TC83 simultaneously, different sites	17D+TC83
	IV	TC83 & 17D on day 3	TC83+3+17D
	V	17D & TC83 on day 3	17D+3+TC83
	VI	TC83 & 17D on day 2	TC83+2+17D
	VII	17D & TC83 on day 2	17D+2+TC83
	VIII	None	TC83
250,000	IX	17D & TC83 simultaneously, same site	17D-TC83
	X	TC83 & 17D on day 3	TC83+3+17D
	XI	17D & TC83 on day 3	17D+3+TC83
	XII	None	TC83

Groups IX, X and XI, given 250,000 median guinea pig intraperitoneal infectious doses ($GPIPID_{50}$) were comparable to Groups I, II and III of the 1969 study.[2/]

When the mean antibody responses of days 14 and 28 postvaccination of Groups IX, X and XI are comparable to results from 1969 if the same criteria are used to evaluate antibody response (SNI $\geq$1.0 was considered a positive response), Table II. A marked interference in YF SN response is seen when TC83 is administered with 17D. However, the interference with the VEE HI response observed in Group XI (Table II) is in marked contrast to that obtained in the previous study,[2/] in which the comparable group of monkeys developed the highest HI titer.

TABLE II. COMPARISON OF 1969 AND 1971 RESPONSES TO 17D AND 250,000 $GPIPID_{50}$ VEE VACCINE

CONDITION	No.	Year	% (MEAN SNI TO 17D) Day 14		Day 28		% (GMT HI TO VEE) Day 14		Day 28	
17D-TC83	I	(1969)	9	(1.7)	55	(2.2)	100	(120)	100	(135)
	IX	(1971)	0		33	(1.6)	100	(57)	100	(64)
TC83+3+17D	II	(1969)	75	(1.4)	100	(1.9)	100	(214)	100	(214)
	X	(1971)	67	(1.5)	67	(2.1)	83	(46)	83	(61)
17D+3+TC83	III	(1969)	92	(1.8)	100	(2.1)	100	(302)	100	(254)
	XI	(1971)	83	(1.6)	67	(1.8)	0		9	(80)

In addition to the antagonistic effect of TC83 to 17D antibody stimulation when the 2 vaccines are administered together, this same effect is seen when TC83 precedes 17D vaccination by 2 or 3 days. This effect also can be seen when 17D precedes TC83 if the TC83 inoculum is increased (Group XI vs. Group I), Table III. That this interference in YF SNI is only a delay in the antibody response and not an inhibition of antibody development can be seen in Table III. When YF SNI is measured 56 days postvaccination, there is no difference in the number of animals responding nor in the SNI titers obtained.

TABLE III. YELLOW FEVER NEUTRALIZATION RESPONSE TO 17D VACCINE[a]

VEE VACCINE DOSE GPIPID$_{50}$	GROUP No.	GROUP Code	NO. RESPONDING/6 (MEAN SNI), BY DAY 7	10	28	56
0	I	17D	4 (2.1)	4 (2.0)	5 (2.0)	6 (4.2)
5,000	II	17D-TC83	1 (1.7)	2 (1.8)	2 (2.0)	6 (3.9)
	III	17D+TC83	4 (2.0)	4 (1.8)	4 (2.1)	6 (3.7)
	IV	TC83+3+17D	4 (1.8)	3 (1.7)	4 (1.8)	6 (3.6)
	V	17D+3+TC83	3 (2.0)	3 (1.7)	6 (1.9)	6 (3.9)
	VI	TC83+2+17D	0	0	3 (1.7)	6 (4.1)
	VII	17D+2+TC83	4 (1.9)	4 (1.8)	6 (2.1)	6 (3.5)
250.000	IX	17D-TC83	0	0	1 (2.2)	6 (4.1)
	X	TC83+3+17D	2 (1.7)	2 (1.8)	3 (2.4)	6 (3.6)
	XI	17D+3+TC83	2 (2.1)	2 (1.9)	3 (1.9)	6 (4.5)

a. Day 0, all <1.7. Day 14 did not differ markedly from day 10 or 28.

The VEE HI and SNI titers are shown in Tables IV and V. The interaction of 17D vaccination with development of TC83-induced antibody response remains inconclusive due the relatively poor response of the TC83 control group (VIII). When the larger TC83 inoculum was used, a greatly decreased antibody response to TC83 was observed when 17D preceded TC83 vaccination (Group XI vs. Group XII), Tables IV and V.

In conclusion, TC83 vaccine interferes with responses to YF through day 28 postimmunization; by day 56 no difference can be seen (Table III). YF vaccine interferes with the VEE HI response when the vaccines are given simultaneously (Group II) and when either precedes the other at the low dose of vaccine; this difference appears to be abolished when the vaccine dose is increased, except for animals receiving 17D before TC83 (Group XI) (Table IV). VEE SNI response is affected similarly by administration of 17D vaccine, although the differences are not quite as apparent (Table V).

TABLE IV. VEE HI RESPONSE OF MONKEYS[a/]

VEE VACCINE DOSE GPIPID$_{50}$	GROUP No.	GROUP Code	NO. RESPONDING/6 (GEOM. MEAN HI TITER) BY DAY 10		14		28		56	
5,000	II	17D-TC83	2	(10)	3	(20)	5	(26)	5	(26)
	III	17D+TC83	4	(24)	4	(48)	5	(61)	5	(121)
	IV	TC83+3+17D	1	(20)	1	(40)	4	(34)	5	(53)
	V	17D+3+TC83	1	(20)	1	(80)	2	(40)	2	(57)
	VI	TC83+2+17D	2	(10)	2	(14)	5	(23)	5	(23)
	VII	17D+2+TC83	0		0		1	(20)	3	(20)
	VIII	TC83	1	(20)	1	(14)	4	(20)	4	(20)
250,000	IX	17D-TC83	4	(57)	6	(57)	5	(71)	5	(90)
	X	TC83+3+17D	3	(20)	5	(71)	5	(61)	6	(61)
	XI	17D+3+TC83	0		0		1	(40)	1	(40)
	XII	TC83	5	(18)	6	(57)	6	(64)	6	(101)

a. Day 0, all <1:10; day 7, occasional response, not shown.

TABLE V. VEE SN RESPONSE OF MONKEYS[a/]

VEE VACCINE DOSE GPIPID$_{50}$	GROUP No.	GROUP Code	NO. RESPONDING/6 (MEAN SNI) BY DAY 10		28		56	
5,000	II	17D-TC83	2	(1.7)	2	(1.7)	4	(2.0)
	III	17D+TC83	4	(1.8)	3	(1.7)	5	(2.1)
	IV	TC83+3+17D	1	(1.7)	2	(2.0)	3	(2.2)
	V	17D+3+TC83	0		1	(1.9)	2	(2.1)
	VI	TC83+2+17D	0		1	(2.2)	3	(1.9)
	VII	17D+2+TC83	1	(1.8)	1	(2.0)	2	(2.0)
	VIII	TC83	4	(1.8)	4	(1.7)	4	(1.7)
250,000	IX	17D-TC83	6	(1.9)	6	(2.6)	6	(3.0)
	X	TC83+3+17D	5	(2.0)	5	(1.9)	5	(2.6)
	XI	17D+3+TC83	2	(1.8)	2	(1.8)	1	(1.9)
	XII	TC83	4	(2.1)	5	(2.1)	6	(2.4)

a. Day 0, all <1.7; days 7 and 14 not shown.

In conclusion, TC83 vaccine interferes with responses to YF through day 28 postimmunization; by day 56 no difference can be seen (Table III). YF vaccine interferes with the VEE HI response when the vaccines are given simultaneously (Group II) and when either procedes the other at the low dose of vaccine; this difference appears to be abolished when the vaccine dose is increased, except for animals receiving 17D before TC83 (Group XI) (Table IV). VEE SNI response is affected similarly by administration of 17D vaccine, although the differences are not quite as apparent (Table V).

<u>Summary, Part II:</u>

TC83 and 17D virus vaccines were administered to monkeys at various times ± 3 days relative to each other. HI and SNI antibody to VEE and SNI antibody

to YF were measured through day 56 postvaccination. A delay in YF SNI antibody response was observed when TC83 vaccination preceded, or was coincident with 17D vaccination and a marked depression in response to TC83 was also observed when 17D preceded TC83 vaccination. It would appear from these results that further studies of live vaccine interactions are warranted.

Publications:

None.

LITERATURE CITED

1. U. S. Army Medical Research Institute of Infectious Diseases. 1 July 1970. Annual Progress Report, FY 1970. p. 121 to 125. Fort Detrick, Maryland.

2. U. S. Army Medical Research Institute of Infectious Diseases. 1 July 1969. Annual Progress Report, FY 1969. p. 171 to 178. Fort Detrick, Maryland.

ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 02:	Prevention and Treatment of Biological Agent Casualties
Work Unit No. 096 02 005:	Studies on Antibody Production and Their Binding Properties
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Division:	Bacteriology
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Author:	Mary H. Wilkie
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

174

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
	DA OL0886	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISB'N INSTR'N	8b. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
71 01 06	D. CHANGE	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	02	005
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	CDOG 1212b(9)			

11. TITLE (Precede with Security Classification Code)*
(U) Studies on Antibody Production and Their Binding Properties in BW Defense Research (U)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*
003500 Clinical Medicine; 004900 Defense; 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
67 10	CONT	DA	C. In-house

17. CONTRACT GRANT

a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER*: NA
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	1	50
	CURRENT 72	1	72

19. RESPONSIBLE DOD ORGANIZATION

NAME* USA Medical Research Institute of Infectious Diseases
ADDRESS* Fort Detrick, Md 21701

RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION

NAME* Bacteriology Division USAMRIID
ADDRESS* Fort Detrick, Md 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME* Wilkie, M. H.
TELEPHONE 301 663-4111 Ext 7341
SOCIAL SECURITY ACCOUNT NUMBER

ASSOCIATE INVESTIGATORS
NAME:
NAME:

DA

21. GENERAL USE
Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Antigens; (U) Antibody; (U) Binding strength; (U) Serology; (U) Military Medicine

23. TECHNICAL OBJECTIVE* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Study kinetics of the immune response and antigen-binding properties of antibodies as a means of assessment of protective efficacy of experimental vaccines and therapeutic antisera against bacterial and viral agents.

24 (U) Establish an arbitrary scale to express binding affinities in place of presently used equilibrium constants, utilizing different biological or physical chemical techniques. Results will be related to protection tests.

25 (U) 70 07 - 71 06 - Kinetics of the primary and secondary immune response of the rabbit have been characterized by quantitative antigen-binding of the immunoglobulin classes involved. The use of the rabbit as an immunological model for evaluation of va[illegible]ne dose and schedule of administration in other hosts is proposed.

[illegible] feasibility of employing an immunological model animal, the rabbit, to evaluate [illegible]n equine encephalitis (EEE) vaccine for human use is currently being explored. Preliminary results indicate that EEE antigens behave in the rabbit like the reference model antigen, BSA.

The application of immune lymphocyte cytotoxicity assay methods to determine immune status in the absence of serological evidence is being evaluated.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 096 02 005: Studies on Antibody Production and Their Binding Properties

Description:

Study kinetics of the immune response and antigen-binding properties of antibodies as a means of assessment of protective efficacy of experimental vaccines or therapeutic antisera against bacterial and viral agents.

Progress, Part I:

Employing bovine serum albumin (BSA) as a classical soluble protein antigen, evaluation of kinetics of the immune response of rabbits by quantitative binding methods has been completed[1/] (USAMRIID Annual Progress Report, FY 1970. p. 127). BSA was selected as the model antigen because it is a foreign, rapidly degraded protein, whose only demonstrable biological effect is immunogenicity. Thereby, normal immune responses of the rabbit to primary and secondary stimulation was established.

In the primary sequence described previously,[1/] antisera from animals immunized with 2 doses of 25 mg each, with or without Freund's adjuvant, were separated into fractions containing major IgM and IgG immunoglobulins; absolute amounts of binding activity of each class were determined in samples drawn at 7-day intervals throughout the antibody synthesizing period. In the primary response, synthesis of IgM became maximal at day 7 and was barely detectable by day 14. The use of Freund's adjuvant did not affect the total amount of IgM synthesized.

In contrast, when no adjuvant was employed, synthesis of specific IgG reached a maximum on day 14, decreasing thereafter at a rate compatible with the 7-day half-life of normal γ globulin. However, following immunization with BSA-adjuvant, specific IgG synthesis continued for 45-60 days. Throughout this period, antibody levels were 25-50% higher than those obtained without adjuvant. Furthermore, the total quantity of IgG synthesized was approximately 100 X greater than that found after immunization with BSA alone.

Eight months to a year later, when rabbits in both groups were producing no detectable circulating antibody, they were restimulated with 10 mg BSA to test residual immunological memory. The kinetics of appearance of IgG and IgM antibodies in sera were followed as in the primary study. Two types of secondary response were observed: In animals

immunized with BSA alone in the primary stage, IgG response was detectable by day 4, increased rapidly to a peak value at day 7, and was sustained through day 28. In addition, IgM appeared at about 7 days, reached a peak on day 14, and disappeared by day 21. It appeared that a "memory"-type IgG response between day 0 and day 7 recycled 10 mg of antigen more efficiently through a new primary response beginning on day 7 than the original dose of 50 mg. Sustained IgG levels appeared to result from reinforcement of anamnestic IgG with new primary IgG by day 14. Normally, primary immunization with 10 mg BSA would produce approximately 10% of these levels. Therefore, we reasoned that increased efficiency of the secondary response was due to "memory" from the primary series a year earlier. Since "memory" is dependent upon the amount of antigen given at that time, it appeared that antigen dosage in the primary exposure controlled the magnitude of the secondary response.

In animals originally immunized with BSA in Freund's adjuvant, a different response was observed. Although anamnestic IgG appeared by day 4, the peak obtained by day 7 was only half that of animals immunized without adjuvant. Low levels of IgM, similar to a primary response in an inexperienced animal, appeared by day 7. The composite IgG response was low suggesting poor "memory," as if the sustained antibody synthesis following primary exposure had prevented establishment of a "memory" reserve. Moreover, the 10-mg dose of BSA appeared to exceed the amount required to saturate memory processes, thereby, leaving some antigen free to initiate a new primary response. Here, also, magnitude of secondary response appeared to be controlled by the primary immunization.

Thus, in this study, the pattern and order of events in the immune response of a well-characterized species, the rabbit, have been followed by quantitative, rather than the usual qualitative, analysis of serum antibodies. These studies and those in existing literature emphasize that the rabbit is the best immunologically calibrated laboratory species. Since the dynamics of immune responses could be monitored by analysis of sequential serum samples, the study further suggested that the rabbit could serve as a reference model for evaluating immune responses in other species, e.g. man. Since man varies immunologically from the rabbit primarily in degree of responsiveness and metabolic rate, the rabbit could be used for prospective studies to determine vaccine dosages and schedules desirable for use in man. Parameters of such studies must include characterization of that IgG level prerequisite for anamnestic memory[2/] (Immunology 9:333, 1965). Furthermore, the nature of response of the rabbit could be used effectively to characterize the chemical nature and clearance of an unknown antigen. Thus, the rabbit can become an analytical immunological model. A manuscript has been written and is being reviewed.

Summary, Part I:

Kinetics of the primary and secondary immune response of the rabbit have been characterized by quantitative antigen binding of the immunoglobulin

classes involved. The use of the rabbit as an immunological model for evaluation of vaccine dose and schedule of administration in other hosts is proposed.

Progress, Part II:

Studies with a standard rabbit model have been initiated in association with Medical Division Project No. FY 71-5, an evaluation of Eastern equine encephalitis (EEE) vaccine in volunteers. Feasibility of projecting vaccine dose and schedule for humans from analysis of rabbit immune responses will be examined by comparing the kinetics of response in rabbits and in volunteers.

Characterization of viral antigens in EEE vaccine by kinetics of rabbit responses has been virtually completed. Four rabbits were immunized with a total of 2.5 ml reconstituted vaccine administered by injection into multiple sites on 2 separate occasions, 7 days apart; at 7-day intervals thereafter their responses were titrated by hemagglutination-inhibition (HI) and complement fixation (CF) techniques. Sera were separated into fractions containing IgM and IgG immunoglobulins by Sephadex G-200 chromatography.

Titers of unfractionated serum were maximal at day 14 and demonstrated a 4-fold decrease by day 28. This result resembled that of the BSA model antigen and indicated that the major antigens had been degraded and were not extensively recycled in the intracellular immune system. By day 36. no further evidence of response to minor, undegraded, recycled antigens have appeared. HI and CF antibodies were demonstrable in both IgM and IgG fractions. Significant IgG activity by day 7 resembled the response to an unconjugated, nontoxic, multisite protein that was probably not extensively degraded extracellularly. Lipid did not influence the response. Absolute antibody content of the sera will be estimated when information regarding quantity of antigen mass in both the vaccine and serological reagent can be obtained.

Some sera from volunteers from the project have been chromatographed and will be analyzed similarly.

Summary, Part II:

The feasibility of employing an immunological model animal, the rabbit, to evaluate EEE vaccines for human use is currently being explored. Preliminary results indicate that EEE vaccine antigens behave in the rabbit like the reference model antigen, BSA.

Progress, Part III:

A feasibility study was initiated to determine if cytolysis of antigen target cells by immune lymphocytes[37] (Cell Bound Antibodies, p. 75) can be used as an indicator system for detecting memory following cessation of

antibody synthesis. Efficacy of a vaccine depends upon its ability to leave active cellular "memory" as well as to induce antibody synthesis. When there is no serological evidence of response, a practical *in vitro* method to determine prior experience with a given antigen without affecting the host would be highly desirable. The assay method using ^{51}Cr-tagged target cells[4] (Immunology 12:525, 1967) was explored because of its simplicity and rapidity. Using goose red cells as antigenic target cells with splenic lymphocytes from immunized inbred C57-BL/6 or DBA mice, conditions of the assay were reproduced and immune cytotoxicity was demonstrated. During these studies, inhibition of cytotoxicity by specific homologous antibody was confirmed[5] (Nature 213:1246, 1967). The possible applications of these findings are being evaluated.

Summary, Part III:

The application of immune lymphocyte cytotoxicity assay methods to determine immune status in the absence of serological evidence is being evaluated.

Publications:

None.

LITERATURE CITED

1. U. S. Army Medical Research Institute of Infectious Diseases. 1 July 1970. Annual Progress Report, FY 1970. p. 127 to 132. Fort Detrick, Maryland.

2. Nossal, G. J. V., C. M. Austin, and G. L. Ada. 1965. Antigens in immunity. VII. Analysis of immunological memory. Immunology 9:333-348.

3. Rosenau, W. 1963. Interaction of lymphoid cells with target cells in tissue culture, p. 75 to 80. *In* B. Amos and H. Koprowski (ed.) Cell Bound Antibodies. Wistar Institute Press, Philadelphia.

4. Holm, G., and P. Perlmann. 1967. Quantitative studies on phytohaemagglutinin-induced cytotoxicity by human lymphocytes against homologous cells in tissue culture. Immunology 12:525-536.

5. Brunner, K. T., J. Mauel, and R. Schindler. 1967. Inhibitory effect of isoantibody on *in vivo* sensitization and on the *in vitro* cytotoxic action of immune lymphocytes. Nature 213:1246-1247.

ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 02:	Prevention and Treatment of Biological Agent Casualties
Work Unit No. 096 02 007:	Evaluation of Experimental Vaccines in Laboratory Animals
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Diseases Frederick, Maryland
Division:	Animal Assessment
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Authors:	Richard O. Spertzel, Lt Colonel, VC Robert W. McKinney, Lt Colonel, MSC
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
	DA OL0893	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
70 07 01	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	02	007
b. ~~CONTRIBUTING~~	62706A	1B662706A096	02	
c. ~~CONTRIBUTING~~	CDOG 1212b(9)			

11. TITLE (Precede with Security Classification Code)* (U) Evaluation of Experimental Vaccines in Laboratory Animals in BW Defense Research (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*
003500 Clinical Medicine; 004900 Defense; 002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
61 10	CONT	DA	C. In-house

17. CONTRACT/GRANT
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER*:
c. TYPE: NA d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	2	40
	CURRENT 72	2	20

19. RESPONSIBLE DOD ORGANIZATION
NAME:* USA Medical Research Institute of Infectious Diseases
ADDRESS:* Fort Detrick, Md 21701
RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
NAME:* Animal Assessment Division USAMRIID
ADDRESS:* Fort Detrick, Md 21701
PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME:* Spertzel, R. O.
TELEPHONE: 301 663-4111 Ext 7244
SOCIAL SECURITY ACCOUNT NUMBER:
ASSOCIATE INVESTIGATORS
NAME: Mason, D. W.
NAME: McKinney, R. W.

21. GENERAL USE
Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Equine encephalomyelitis (VEE); (U) Epizootic; (U) Central America; (U) Attenuated virus vaccine; (U) Vaccines; (U) Immunization; (U) Military medicine

23. TECHNICAL OBJECTIVE.* 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Evaluate experimental vaccines or antigens in laboratory and other animals before use in man. Assist in control of an epizootic.

24 (U) Promising vaccines are given to laboratory animals and safety tested prior to administration to man. Supply vaccine and technical assistance to conduct a vaccination program in order to create a barrier of immune horses and thereby curb the spread of the disease in these animals.

25 (U) 70 07 - 71 06 - In early May 1969, the first severe epizootic of Venezuelan equine encephalomyelitis (VEE) erupted on the Pacific Coastal Plain of Guatemala, adjacent to the El Salvador border. From this area, the disease rapidly swept westward and northward in Guatemala, and eastward through El Salvador, Honduras and Nicaragua before subsiding in October. In June 1970, the disease appeared in Mexico near the Guatemalan border, and by July had spread westward about 200 miles. In August, cases were reported in Guanacaste Province, Costa Rica.

An attenuated, live-virus vaccine was used to aid in the control of these epizootics. Vaccination of the horse population at the periphery of the epizootic area was accomplished to create an immune barrier in an attempt to limit the spread of VEE infection. Vaccination of horses also was practiced within the epizootic area to control the disease. Pre- and postvaccinal sera were collected from 157 Equidae in Mexico. A 96% seroconversion rate was obtained.

Studies previously reported here on experimental toxoids are now reported under Work Unit 096 02 800.

Publications: Amer. J. Trop. Med. Hyg., 1971, In press.
Proc. U. S. Animal Health Assoc., 1971, In press.

*Available to contractors upon originator's approval

DD FORM 1498, 1 MAR 68 — PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 096 02 007: Evaluation of Experimental Vaccines in Laboratory Animals

Description:

Evaluate experimental vaccines in laboratory and other animals. Assist in control of an epizootic.

Progress and Summary, Part I:

Testing of experimental staphylococcal enterotoxoids, previously reported here, are now included under Work Unit 096 02 800.

Progress, Part II:

In early May 1969, a severe epizootic of Venezuelan equine encephalomyelitis (VEE) erupted on the Pacific Coastal Plain of Guatemala, adjacent to the El Salvador border. From this area, the disease rapidly swept westward and northward in Guatemala, and eastward through El Salvador, Honduras and Nicaragua before subsiding in October of the same year[1/] (CES Annual Report, FY 1969, p. 163). In June 1970, the disease appeared in Mexico near the Guatemalan border, and by July had spread westward about 200 miles. In August cases were reported in Guanacaste Province, Costa Rica. To aid in control of the disease, an attenuated, live virus VEE vaccine (TC-83) and technical assistance for the administration of this vaccine were provided to the governments of the countries involved.

It should be noted that at the time of the initial request for assistance, the extent, location or duration of the epizootic was not known; nor was it certain that the epizootic was due to Venezuelan-type virus. However, based on information available from epidemiologic and ecologic surveys conducted previously by several groups, it seemed reasonable to assume we were dealing with VEE.

To abort the epizootic, it was crucial that a well-organized VEE vaccination program be conducted. Since vaccinated animals are not protected until 7-10 days after inoculation, there was no hope of stopping the spread of the epizootic by immunizing animals in areas where the disease already was occurring. For the establishment of barriers of immune animals to contain the epizootic, it would be necessary to inoculate Equidae approximately 2 weeks before the arrival of the disease. We were, however, unable to evaluate the ability of the countries' governments to carry out the massive vaccination programs within the required period of time.

Despite unanswered questions about (1) the ability to start the program, (2) the willingness of the national governments to participate in an approach that had never been tried in the control of VEE, and (3) the rate of spread of disease from the init al focus, we elected to rely on immune barriers to contain the disease. It was reasoned that this approach would permit the most effective use of the vaccine, since the animals to be vaccinated were some distance from the center of the epizootic and thus could be expected to be free of disease at the time of inoculation, and have sufficient time to be rendered immune before the arrival of virulent virus. The massive vaccination program would also lessen the number of susceptible animals in the population, thereby slowing the rate of disease transmission.

The vaccination campaigns in both Guatemala and El Salvador began on 15 July 1969. Both governments were well-organiz[illegible]nd the campaigns were fully operational in the subsequent 3-4 days. This [illegible]rticularly encouraging in El Salvador, since it was at war with Hondur[illegible]

Delineation of the epizootic area was co[illegible]d by the lack of accurate case-reporting. In some cases, false reports [illegible]EE were submitted in an attempt to obtain vaccine. In remote areas, horses died without the owners' knowledge, since animals were allowed to roam freely. The uncertainty of locating diseased animals caused some concern relative to use of the vaccine. In particular, we were concerned with the reaction of owners whose horses were infected at the time of inoculation and died subsequent to vaccination. This did occur, but did not affect acceptance of the vaccine. Another concern was that, because it was necessary to accomplish multiple inoculations with the same syringe, the transmission of Equine Infectious Anemia, or of VEE, was a real possibility should an infected horse be included in the group being vaccinated. The governments involved were aware of such possibilities, but concluded that the benefits occurring from mass vaccinations outweighed such dangers. Use of the jet gun would have eliminated the transmission problem, but it is our opinion that, considering field conditions and the intractability of many of the animals, inoculations could not have been accomplished as efficiently as with conventional needle and syringe. It should also be noted that the jet gun employed for vaccinating humans requires modification for inoculating horses.

The vaccination campaigns represented the first large-scale field use of the attenuated vaccine for immunization of horses. The urgent need to protect the equine populations, coupled with a variety of other factors, precluded establishing controlled studies for assessment of vaccine efficacy. However, observations made in the field do serve to indicate that in field use, the vaccine was effective. Seven to 10 days after vaccination, even on ranches where active cases were occurring, all equine cases of VEE ceased. Complete protection occurred in local areas, e.g., valleys or large ranches within the eventual epizootic area, where vaccination was completed 7-10 days prior to spread of the disease into the area. In Guatemala, an immune barrier of vaccinated horses, about 50 km wide, was established on the Pacific Coastal Plain. This barrier prevented the spread of VEE westward. In the upper and lower Motagua Valley, vaccination also was successfully employed to halt the disease. Similarly, by the use of a massive VEE vaccination program,

the disease was confined to the Pacific Coastal Plain of Honduras and contained in the southwestern corner of Nicaragua.

Although the 1969 epizootic was contained, the disease erupted again in 1970 in Guanacaste Province of Costa Rica. It is postulated that the infection originated in Nicaragua. If this is correct, VEE infection breached a barrier zone on which more than 90% of the Equidae had been vaccinated the previous summer, and which was free of reported cases of VEE in both 1969 and 1970. The source of the epizootic in Mexico is not known.

It must be emphasized that the vaccination program employed an attenuated, live-virus vaccine. While this product has proven to be both safe and efficacious in the vaccination programs reported here, a different picture emerges for inactivated preparations of virulent VEE virus. In the spring of 1970, an inactivated VEE vaccine, prepared commercially in Central America in accordance with standards established for Eastern and Western equine encephalomyelitis vaccines in the United States, was used in Nicaragua. Concurrent with the use of this vaccine, encephalitis appeared in horses, and the deaths were traced to the vaccine. This corresponds to findings made during the 1950's: that laboratory personnel, inoculated with inactivated VEE vaccine prepared in essentially the same manner as the Central American product, developed VEE. Although standard tests in both situations indicated the absence of residual live virus, use of the vaccine resulted in clinical disease. The inability to produce an inactivated vaccine that was both safe and effective prompted the development of the attenuated VEE vaccine that we used in Central America.

During the Mexican Control Program, August to October 1970, attempts were made to collect sera from 275 Equidae prior to and $\geq$ 30 days after vaccination. These Equidae were vaccinated by a regular Mexican vaccinating team and were located at the periphery of the epizootic where no VEE was known to be occurring. Of these 275, 163 paired samples (136 horses, 24 mules, 3 burros) were obtained. Six horses had preexisting antibody titers, Table I. Hemagglutination-inhibition (HI) titers of 4 mules and 3 horses went from $<$ 1:10 to 1:10 only; all other animals with pretiters of $<$ 1:10-$>$ 1:20 had maximum titers of 1:320, at 30 days.

TABLE I. HI RESPONSE OF EQUIDAE 1-1/2 MON POSTVACCINATION WITH ATTENUATED VEE (TC-83), MEXICO, 1970

RECIPROCAL HI[a] TITER	HORSES Female	HORSES Male	MULES Female	MULES Male	BURROS Male	TOTAL EQUIDAE
10	0	3	0	4	0	7
20	4	6	4	2	1	17
40	12	27	4	2	1	46
80	13	17	3	1	0	34
160	23	17	2	2	1	45
320	4	3	0	1	0	8
	56	73	13	12	3	157
Median titer	80	80	40	30	40	80
Mean titer	92	63	47	36	49	67

a. 6 horses had preexisting titers of ≥ 1:20 and were excluded from study; all other prevaccination titers were < 1:10.

Summary, Part II:

In early early May 1969, the first severe epizootic of VEE erupted on the Pacific Coastal Plain of Guatemala, adjacent to the El Salvador border. From this area, the disease rapidly swept westward and northward in Guatemala, and eastward through El Salvador, Honduras and Nicaragua before subsiding in October 1969. In June 1970, the disease appeared in Mexico near the Guatemalan border, and by July had spread westward about 200 miles. In August, cases were reported in Guanacaste Province, Costa Rica. An attenuated, live-virus vaccine was used to aid in the control of these epizootics. Vaccination of the horse population at the periphery of the epizootic area was accomplished to create an immune barrier in an attempt to limit the spread of VEE infection. Vaccination of horses also was practiced within the epizootic area to control the disease.

Presentations:

1. Spertzel, R. O. 1970. VEE, a disease on the move. Presented at Annual Meeting of U. S. Animal Health Association, Philadelphia, Pa. 21 Oct 1970.

2. Spertzel, R. O. 1971. VEE--the disease and the recent outbreaks in Central and South America. Presented at Foreign Animal Diseases Seminar, National Animal Disease Laboratory, Ames, Iowa 15 Jan 1971.

3. Spertzel, R. O. 1971. Current status of live attenuated VEE virus vaccine--its use in Central America and Mexico. Presented at Annual Southwestern Conference on Diseases in Nature Transmissible to Man, College of Veterinary Medicine, Texas A&M University, College Station, Texas 20-21 May 1971.

Publications:

1. Spertzel, R. O., and R. W. McKinney. 1971. Venezuelan equine encephalomyelitis in Central America and Mexico. Am. J. Trop. Med. Hyg. (In press).

2. Spertzel, R. O., and R. W. McKinney. 1971. VEE, a disease on the move. Proceedings, Annual Meeting, U. S. Animal Health Assoc. (In press).

LITERATURE CITED

1. McKinney, R. W. Dec 1969. Venezuelan equine encephalitis in Central America: A progress report. p. 163 to 170. In Commission on Epidemiological Survey, Annual Report to the Armed Forces Epidemiological Board FY 1969, Fort Detrick, Maryland.

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ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 02:	Prevention and Treatment of Biological Agent Casualties
Work Unit No. 096 02 008:	Evaluation of Efficacy of Combined Antigens in Man
Reporting Installation:	U.S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Divisions:	Medical and Virology
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Authors:	Peter J. Bartelloni, Lt Colonel, MC Nemesio M. Francisco, Major, MC Francis E. Cole, Jr., Ph.D. Helen H. Ramsburg, B.S.
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
	DA OL0894	71 07 06	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 01 06	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	02	008
b. CONTRIBUTING				
c. ~~[illegible]~~	CDOG 1212b(9)			

11. TITLE (Precede with Security Classification Code)* (U) Evaluation of Efficacy of Combined Antigens in Man in BW Defense Research (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*
003500 Clinical Medicine; 004900 Defense; 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
69 02	CONT	DA	C. In-house

17. CONTRACT GRANT
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER*: NA
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.:

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	2	90
	CURRENT 72	2	110

19. RESPONSIBLE DOD ORGANIZATION
NAME:* USA Medical Research Institute of Infectious Diseases
ADDRESS:* Fort Detrick, Md 21701

RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
NAME:* Medical Division USAMRIID
ADDRESS:* Fort Detrick, Md 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME:* Bartelloni, P. J.
TELEPHONE: 301 663-4111 Ext 6135
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Francisco, N. M.
NAME: DA

21. GENERAL USE
Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Vaccines; (U) Immunization; (U) Encephalitis, equine (EEE, WEE); (U) Military Medicine

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Test and evaluate combinations of vaccines in man.

24 (U) After combination antigens have been safety tested and evaluated in laboratory animals, they are given to man.

25 (U) 70 07 - 71 06 - Volunteers were administered 2 doses of 0.5 ml each of combined inactivated Eastern and Western equine encephalitis vaccine 28 days apart to obtain additional information on serological responses. The majority of individuals developed significant Western neutralizing antibody (more than 1.7 logs) by day 28. All subjects achieved significant neutralizing antibody on days 42, 56, and 90. Only one of 11 and 2 of 12 subjects had inadequate neutralizing antibody on days 180 and 270 respectively.

All subjects developed significant Eastern virus neutralizing antibody on days 42 and 56 and the majority of the subjects maintained significant neutralizing antibody levels through day 270.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 096 02 008: Evaluation of Efficacy of Combined Antigens in Man

Description:

Test and evaluate combinations of vaccines in man.

Progress:

Evaluation of Combined Western Equine Encephalitis (WEE) Vaccine, Formalin-Inactivated, Tissue Culture Origin, Lot 1-1967 and Eastern Equine Encephalitis (EEE) Vaccine, Formalin-Inactivated, Tissue Culture Origin, Lot 1-1966 in volunteers (Medical Division Protocol FY 70-6): Clinical, serological responses and laboratory determinations on 16 volunteers administered combined, inactivated WEE and EEE vaccines has been reported previously[1,2] (USAMRIID Annual Progress Reports, FY 1969, p. 87, FY 1970, p. 145). Neutralizing antibody responses to Western equine encephalitis virus were unsatisfactory when Western vaccine was administered in combined form with Eastern vaccine. The reason for the unsatisfactory responses of neutralizing antibody to Western virus was not known, but was felt to be due to individual or laboratory variation or to the manner in which the vaccines were reconstituted. Therefore, an additional study to evaluate the serological responses to combined Western and Eastern vaccines was initiated in 16 healthy volunteers not previously immunized with EEE and WEE vaccine and having no history of infection with these viruses. Each volunteer was administered 2 doses of 0.5 ml each of combined Eastern and Western vaccine 28 days apart. Blood for neutralizing antibodies was obtained prior to vaccination and on days 28, 42, 56, 90, 180, and 270. The results are shown in Tables I and II (underlined figures indicate $\geq$ 1.7 logs).

As shown in Table I, the majority of subjects developed significant neutralizing antibody to WEE virus by day 28 with a mean LNI ($\log_{10}$ serum neutralization index) of 1.9. All subjects developed adequate antibody to Western virus on days 42, 56, and 90 with mean LNI of 2.9, 2.8 and 2.5 respectively. On days 180 and 270, 10 of 11 and 10 of 12 subjects maintained significant titers, with mean LNI of 2.4 and 2.3.

TABLE 1. WEE NEUTRALIZING ANTIBODY RESPONSES[a/] OF VOLUNTEERS ADMINISTERED 2 DOSES OF COMBINED WEE AND EEE VACCINES ON DAYS 0 AND 28

VOLUN-TEERS	LOG_{10} SNI BY DAY POSTVACCINATION					
	28	42	56	90	180	270
GLG	2.6	3.2	3.3	3.2	2.8	2.8
GMG	2.0	3.3	2.6	2.1	ND[b/]	ND
BEG	1.2	3.2	2.5	2.5	2.0	2.2
RWH	1.4	3.0	3.1	2.6	2.9	2.7
WNJ	1.9	2.2	2.1	2.0	2.1	2.4
KRK	2.0	2.7	2.6	2.4	2.9	1.9
REL	2.3	2.5	2.8	2.1	ND	ND
DAL	1.1	2.3	2.3	2.0	2.0	2.3
RLM	1.2	3.4	2.9	2.5	1.9	1.6
DMP	2.1	2.2	2.1	2.3	1.5	1.6
RDP	2.3	3.3	3.3	2.8	3.4	3.0
GWR	2.3	3.4	3.4	3.1	ND	ND
CTR	1.8	3.1	2.6	2.3	ND	ND
JNS	2.4	3.3	2.8	2.7	2.3	1.8
WET	2.5	3.3	2.5	2.3	ND	2.0
SSW	2.8	3.7	3.8	3.6	2.9	2.8
MEAN	1.9	2.9	2.8	2.5	2.4	2.3
No. _ 1.7/ TOTAL	12/16	16/16	16/16	16/16	10/11	10/12
%	75	100	100	100	91	83
RANGE	(1.1-2.8)	(2.2-3.7)	(2.1-3.8)	(2.0-3.6)	(1.5-3.4)	(1.6-3.0)

a. No subject had demonstrable neutralizing antibody on day 0.
b. ND = Not done.

TABLE II. EEE NEUTRALIZING ANTIBODY RESPONSES[a] OF VOLUNTEERS ADMINISTERED 2 DOSES OF COMBINED WEE AND EEE VACCINES ON DAYS 0 AND 28

VOLUNTEERS	LOG_{10} SNI BY DAY POSTVACCINATION 28	42	56	90	180	270
GLG	1.3	2.4	2.3	1.9	1.8	1.8
GMG	2.3	3.0	2.7	2.4	ND[b]	ND
BEG	1.1	3.1	2.5	2.0	1.9	1.8
RWH	1.8	3.1	2.8	2.4	2.4	2.4
WNJ	1.3	2.4	1.9	2.3	1.5	1.7
KRK	1.5	2.2	2.3	2.5	2.0	2.2
REL	2.2	3.0	2.6	2.8	ND	ND
DAL	1.5	2.0	2.4	1.8	1.6	1.4
RLM	1.0	2.9	2.3	2.3	1.6	1.6
DMP	1.5	2.7	3.0	2.4	1.8	1.6
RDP	1.9	3.2	2.5	2.3	2.5	2.4
GWR	2.4	3.1	3.0	2.9	ND	ND
CTR	1.5	2.6	2.6	2.1	ND	ND
JNS	0.5	1.9	1.8	1.4	1.9	1.8
WET	2.0	3.0	3.2	2.8	ND	1.8
SSW	2.6	2.8	2.9	2.3	2.4	2.5
MEAN	1.7	2.7	2.5	2.3	1.9	1.9
No. ≥1.7/ TOTAL	7/16	16/16	16/16	15/16	8/11	9/12
%	44	100	100	94	73	75
RANGE	(0.5-2.6)	(1.9-3.2)	(1.8-3.2)	(1.4-2.9)	(1.5-2.5)	(1.4-2.5)

a. No subject had demonstrable neutralizing antibody on day 0.
b. ND = Not done.

The majority of subjects failed to achieve significant EEE neutralizing antibody by 28 days after the first dose of combined vaccine. The mean LNI 28 days after the first dose of combined vaccine was 1.7 logs. However by day 42 the mean LNI was 2.7 logs and all subjects developed significant EEE neutralizing antibody. The mean LNI remained at significant levels through day 270. A decrease in EEE antibody below 1.7 logs occurred in 3 vaccinees on day 180 and in one subject on day 270.

A combined Eastern and Western inactivated vaccine was evaluated in 16 volunteers to compare the efficacy of the serological responses with a similar previous study (Medical Division Protocol FY 69-6). The mean LNI to Western antibody up to day 270 and the number of vaccinees responding were greater than in the previous study (Table III). The mean EEE LNI and the number of subjects with significant neutralizing antibody titers up to day 270 did not show a significant difference between study FY 69-6 and the present study by X^2 analysis.

TABLE III. COMPARISON OF RESPONSES TO COMBINED WEE-EEE VACCINES GIVEN IN 2 DOSES FROM MEDICAL DIVISION PROJECTS FY 69-6 and FY 70-6

	WEE				EEE			
DAY	69-6[2]		70-6		69-6[2]		70-6	
POST VACC.	% ≥1.7 logs	mean LNI	% ≥1.7 logs	mean LNI	% ≥1.7 logs	mean LNI	% ≥1.7 logs	mean LNI
14	27	1.4	ND	ND	27	1.5	ND	ND
28	19	1.4	75	1.9	50	1.7	44	1.7
42	50	1.7	100	2.9	100	2.7	100	2.7
56	84	1.9	100	2.8	100	2.5	100	2.5
90	84	1.7	100	2.5	88	2.2	94	2.3
180	56	1.7	91	2.4	50	1.8	73	1.9
270	56	1.8	83	2.3	63	1.8	75	1.9
360	38	1.6			38	1.5		

Summary:

Combined EEE and WEE vaccines were administered to 16 volunteers to verify apparent poor responses shown in a previous study. The results for WEE antibodies were improved and those for EEE were similar.

Publications:

None

LITERATURE CITES

1. U. S. Army Medical Research Institute of Infectious Diseases. 1 July 1969. Annual progress report, FY 1969. p. 87 to 102. Fort Detrick, Maryland.

2. U. S. Army Medical Research Institute of Infectious Diseases. 1 July 1970. Annual progress report, FY 1970. p. 145 to 154. Fort Detrick, Maryland.

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ANNUAL PROGRESS REPORT

Project No. 3A61101A91C:	Independent Laboratory In-house Research
Task No. 3A61101A91C 00: (1B662711A096 02)	(Prevention and Treatment of Biological Agent Casualties)
Work Unit No. 91C 00 133: (096 02 009)	Studies with Human Diploid Cell Cultures
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Division:	Virology
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Author:	Albert T. McManus, Captain, MSC
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION: DA OL0901
2. DATE OF SUMMARY: 71 07 01
REPORT CONTROL SYMBOL: DD DR&E(AR)636

3. DATE PREV SUM'RY: 70 07 21
4. KIND OF SUMMARY: D. CHANGE
5. SUMMARY SCTY: U
6. WORK SECURITY: U
7. REGRADING: NA
8a. DISB'N INSTR'N: NL
8b. SPECIFIC DATA-CONTRACTOR ACCESS: ☒ YES ☐ NO
9. LEVEL OF SUM: A. WORK UNIT

10 NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A61101A91C	00	133
b. ~~CONTRIBUTING~~	62711A	1B662711A096	02/009	
c. ~~CONTRIBUTING~~	CDOG 1212b(9)			

11. TITLE: (U) Studies with Human Diploid Cell Cultures for Production of Military Vaccines (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical Medicine; 004900 Defense; 010100 Microbiology

13. START DATE: 69 11
14. ESTIMATED COMPLETION DATE: CONT
15. FUNDING AGENCY: DA
16. PERFORMANCE METHOD: C. In-house

17. CONTRACT GRANT
a. DATES/EFFECTIVE:
EXPIRATION:
b. NUMBER:
c. TYPE: NA
d. AMOUNT:
e. KIND OF AWARD:
f. CUM. AMT.:

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	2	40
	CURRENT 72	1	40

19. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, Md 21701
RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
NAME: Virology Division USAMRIID
ADDRESS: Fort Detrick, Md 21701
PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: McManus, A. T.
TELEPHONE: 301 663-4111 Ext 7241
SOCIAL SECURITY ACCOUNT NUMBER
ASSOCIATE INVESTIGATORS
NAME:
NAME:
DA

21. GENERAL USE: Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Diploid cell culture; (U) Immunization; (U) Vaccines; (U) WI-38 (U) Military Medicine;

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Evaluate human diploid cell cultures for use as substrate for preparation of viral and rickettsial vaccines.

24 (U) The human diploid cell, WI-38, will be studied to establish the technical requirements to produce and quality control cell strain substrates for human vaccines.

25 (U) 70 07 - 71 06 - Growth and characterization of human diploid cell strain WI-38 is presently at the seed stock development phase.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 3A61101A91C: Independent Laboratory In-house Research

Task No. 3A61101A91C 00: (Prevention and Treatment of Biological Agent
(1B662711A096 02) Casualties)

Work Unit No. 91C 00 133: Studies with Human Diploid Cell Cultures
(096 02 009)

Description:

Evaluate human diploid cell cultures for use as substrate for preparation of viral and rickettsial vaccines.

Progress:

A separate area has been organized for the production and characterization of human diploid cells. This area meets U. S. Public Health Service requirements for the propagation of cells to be used in vaccine production. The human diploid cell area is presently capable of growing and maintaining cells without contact with other cell culture areas.

Training with the WI-38 cell strain, a diploid cell derived from a fetal human lung, is well underway for the technician assigned to the area. Training has consisted of aseptic manipulation of cultures (without mouth pipetting) using static monolayer and roller bottle culture techniques.

Efforts have been initiated to purchase a single lot of fetal bovine serum free of detectable cytopathic and noncytopathic adventitious agents. Upon completion of the purchase, frozen seed stock of WI-38 at passage 14 (ATCC) will ge grown to produce a USAMRIID stock of WI-38 cells. The cells will be refrozen at approximately passage 20. This seed will consist of approximately 250 ampoules. The expected date of seed production is July 71.

Upon completion of this production, the seed will be characterized to present literature values for WI-38. Concurrent with characterization, virus susceptibility studies and development of an inactivated group A arbovirus vaccine will be initiated.

Summary:

Growth and characterization of the human diploid cell strain WI-38 is presently at the seed stock development phase.

Publications:

None

BODY OF REPORT

Project No. 3A61101A91C: Independent Laboratory In-house Research

Task No. 3A61101A91C 00: (Prevention and Treatment of Biological Agent (1B662711A096 02) Casualties)

Work Unit No. 91C 00 133: Studies with Human Diploid Cell Cultures (096 02 009)

Description:

Evaluate human diploid cell cultures for use as substrate for preparation of viral and rickettsial vaccines.

Progress:

A separate area has been organized for the production and characterization of human diploid cells. This area meets U. S. Public Health Service requirements for the propagation of cells to be used in vaccine production. The human diploid cell area is presently capable of growing and maintaining cells without contact with other cell culture areas.

Training with the WI-38 cell strain, a diploid cell derived from a fetal human lung, is well underway for the technician assigned to the area. Training has consisted of aseptic manipulation of cultures (without mouth pipetting) using static monolayer and roller bottle culture techniques.

Efforts have been initiated to purchase a single lot of fetal bovine serum free of detectable cytopathic and noncytopathic adventitious agents. Upon completion of the purchase, frozen seed stock of WI-38 at passage 14 (ATCC) will ge grown to produce a USAMRIID stock of WI-38 cells. The cells will be refrozen at approximately passage 20. This seed will consist of approximately 250 ampoules. The expected date of seed production is July 71.

Upon completion of this production, the seed will be characterized to present literature values for WI-38. Concurrent with characterization, virus susceptibility studies and development of an inactivated group A arbovirus vaccine will be initiated.

Summary:

Growth and characterization of the human diploid cell strain WI-38 is presently at the seed stock development phase.

Publications:

None

ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 02:	Prevention and Treatment of Biological Agent Casualties
Work Unit No. 096 02 010:	Role of Secretory Immunoglobulin in Host Immunity
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Division:	Bacteriology Division
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Author:	Stanley G. Rabinowitz, Major, MC
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA OL0903	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
70 09 30	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	02	010
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1212b(9)			

11. TITLE (Precede with Security Classification Code)*
(U) Role of Secretory Immunoglobulin in Host Immunity to Militarily Important Diseases (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*
003500 Clinical Medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
70 09	CONT	DA	C. In-house

17. CONTRACT/GRANT
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER:* NA
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	2	70
	CURRENT 72	1	113

19. RESPONSIBLE DOD ORGANIZATION
NAME:* USA Medical Research Institute of Infectious Diseases
ADDRESS:* Fort Detrick, Md 21701
RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
NAME:* Bacteriology Division USAMRIID
ADDRESS:* Fort Detrick, Md 21701
PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME:* Rabinowitz, S. G.
TELEPHONE: 301 663-4111 Ext 7341
SOCIAL SECURITY ACCOUNT NUMBER
ASSOCIATE INVESTIGATORS
NAME:
NAME: DA

21. GENERAL USE
Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Immunoglobulin; (U) Secretory antibody; (U) Delayed hypersensitivity; (U) Humoral antibody; (U) Vaccines; (U) Military Medicine

23. TECHNICAL OBJECTIVE.* 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) To evaluate the relationship of secretory and humoral antibodies to protection afforded by vaccines administered by various routes.

24 (U) Develop an animal model for evaluating efficacy of local secretory antibody in protecting a host from respiratory-acquired infection.

25 (U) 70 09 - 71 06 - An animal model has been developed using the guinea pig to evaluate local respiratory antibody response to vaccine and/or challenge with microorganisms. At present, local antibody has only been demonstrated in animals vaccinated and challenged intraperitoneally with Q fever. Further studies are in progress to evaluate this finding.

A technique for obtaining immunocompetent cells from the peritoneum of guinea pigs has been employed to transfer delayed hypersensitivity. Work is now in progress to assess the protective role of both hyperimmune sera and cells in infection with guinea pigs with VEE and Q fever.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 096 02 010: Role of Secretory Immunoglobulin in Host Immunity

Description:

To evaluate the relationship of secretory and humoral antibodies to protection afforded by vaccines administered by various routes.

Progress, Part I:

Recent progress in characterizing antibodies has led to the establishment of 5 major classes of immunoglobulins. These are IgG, IgM, IgA, IgD and IgE. The first 3 are the major components of circulating, or humoral, antibody. Most antibacterial and antiviral activity is contained in these fractions. IgA, in addition, is the major immunoglobulin present in external secretions, i.e. saliva, colostrum, respiratory tract secretions, etc.[1/] (Adv. Immun. 9:1, 1968). In these secretions, IgA exists in a unique form; it is composed of 7S IgA produced locally by plasma cells in close approximation to the epithelial surface and a secretory component made in the epithelial cell.[1/]

A review of the literature indicates that a number of factors may be involved in stimulating formation of secretory immunoglobulin, e.g. route of inoculation, dose of antigen, and character of the antigen (live or killed)[2/] (Infect. Immun. 2:29, 1970). It should be noted that, with the exception of studies employing Francisella tularensis[3/] (J. Immun. 98:171, 1967), investigations of IgA have employed microorganisms which may be found in the population at large, e.g. polio, rhinoviruses, etc. Thus, evaluation of the significance of IgA in resisting infection is frequently complicated by the possibility of prior experience. Therefore, an investigation was undertaken to evaluate those factors involved in producing secretory IgA when vaccines and/or virulent organisms not commonly encountered were employed, e.g. Coxiella burneti and Venezuelan equine encephalomyelitis (VEE) viruses. Furthermore, it was proposed to evaluate whether secretory immunoglobulin, when present, had a role in protecting the host from infection.

An animal model employing the guinea pig was developed for obtaining tracheobronchial washings (TBW) for antibody activity determinations. Guinea pigs were sacrificed by intraperitoneal (IP) administration of Lethal (Eli Lilly and Company, Indianapolis, Indiana). A midline incision was made in the neck and the trachea was cannulated. The trachea was washed with 1 ml aliquots of Hank's Balanced Salt Solution (without phenol) and the return was collected in a sterile test tube: 10 ml were infused of which approximately 8 ml were recovered. Samples grossly contaminated with blood were discarded. After centrifugation, the remaining samples were evaluated

for contamination with erythrocytes; an observation of no contamination was confirmed by Lowry protein determinations on each supernatant fluid.

Tracheobronchial washings were collected from guinea pigs immunized in the following manner. One group was prepared with 200 µg bovine gamma globulin (BGG) in complete Freund's adjuvant by footpad injection and another with 300 µg BGG alone instilled directly into the trachea after the animals were anaesthetized IP with Surital (Parke-Davis Co., Detroit, Michigan). Subsequently, TBW and serum were obtained at 7-10-day intervals for 35 days. Both serum and TBW were titrated for hemagglutinating (HA) and complement fixing (CF) antibodies. Antibody could not be detected in either serum or TBW of animals administered 300 µg BGG intratracheally (IT). As expected, animals inoculated via the footpad had serum HA titers >1:2000 in 3 weeks; however, HA or CF antibody was not detected in their TBW.

Next, the effect of living, attenuated VEE vaccine (TC-83) was evaluated. A group of guinea pigs was immunized with 1000 median guinea pig intraperitoneal immunizing doses of TC-83 by either the IP or IT route. TBW and sera were obtained serially at 7-day intervals over a 30-day period. Each was tested by hemagglutination inhibition (HI) and neutralization tests for antibody activity. Neutralizing antibody was assessed by a modification of the plaque reduction method using constant amounts of serum and varying dilutions of virus. A neutralization index was then computed.

Antibody could not be detected in the TBW regardless of the route of administration. In contrast, high serum titers of antibody by HI and neutralization tests were found in all animals.

It appeared from these studies that detectable amounts of local antibody were not produced in the respiratory tract of guinea pigs in response to an inert antigen (BGG) or a live, replicating antigen (TC-83).

Finally, the response following immunization and challenge with Q fever rickettsiae was investigated. Guinea pigs were immunized IP with Phase II Henzerling strain vaccine. Two weeks later they were challenged IP with virulent Phase I C. burneti. TBW and sera were collected 11 days following challenge and were tested for antibody content (Table I). Postchallenge CF titers of sera were high in all animals. In contrast to preceding studies, antibody was detected in TBW from guinea pigs following this challenge. CF titers were low; no microagglutinins were detected. It is of interest to note that the highest incidence of response occurred in animals immunized with the smallest antigenic mass. It is possible that the TBW antibody activity is one manifestation of a booster response to challenge. It would be expected that animals receiving the smallest primary stimulus would show the greatest booster response. Confirmatory studies are currently in progress.

TABLE I. SERUM AND TBW ANTIBODY RESPONSES OF IMMUNIZED AND NONIMMUNIZED GUINEA PIGS 11 DAYS FOLLOWING CHALLENGE WITH PHASE I COXIELLA BURNETI.

	SERUM ANTIBODY			TBW ANTIBODY		
VACCINE[a/] DILUTION	Responders Tested	Mean Titers[b/] CF	Mean Titers[b/] Microagg.	Responders Tested	Mean Titer[b/] CF	Mean Titer[b/] Microagg.
1:5	5/5	512	4	1/5	2	Neg
1:25	5/5	512	8	4/5	4	Neg
1:125	5/5	512	4	5/5	4	Neg
1:625	5/5	512	8	5/5	4	Neg
No vaccine	5/5	512	8	1/5	2	Neg

a. Phase II, Henzerling strain Q fever vaccine (WRAIR, Lot DP-7); 0.5 ml injected IP.

b. Mean reciprocal titer.

Summary, Part I:

An animal model has been developed using the guinea pig to evaluate local respiratory antibody response to vaccine and/or challenge with microorganisms. At present, local antibody has only been demonstrated in animals vaccinated and challenged IP with Q fever. Further studies are in progress to evaluate this finding.

Progress, Part II:

Work by MacKaness and others[4, 5/] (J. Infect. Dis. 123:439, 1971; J. Exp. Med. 132:1035, 1970) has shown that microorganisms capable of surviving within phagocytic cells of the reticuloendothelial system, viz. mycobacteria, Burcella, Salmonella, viruses, are primarily eliminated from the host by interaction of immunocompetent lymphocytes with macrophages. This interaction produces an "activated" macrophage with significantly enhanced antimicrobial properties. Antibody alone does not seem capable of protecting the host from these intracellular organisms.

Preliminary experiments have been performed with guinea pigs immunized with 200 µg BGG in complete Freund's adjuvant by footpad injection.

Peritoneal exudate cells (PE) were recovered following IP injection by Bayol-F 10-13 days after immunization. Approximately 9 x 10^7 PE cells (60% lymphocytes, 40% macrophages) were obtained. The cells were centrifuged, washed several times and finally resuspended in Hank's Balanced Salt Solution (HBSS) to a concentration of 2 - 2.5 x 10^8 cells/5 ml. An aliquot of the pooled cell suspension was tested for antibody activity by the HA test. These PE cells were then injected IP into nonimmune guinea pigs; 36 hr later, these adoptively immunized animals were tested for cutaneous hypersensitivity. The skin test dose employed was 10 μg BGG/0.1 ml HBSS. Control injections were 0.1 ml of HBSS were administered on the contralateral side of the shaved abdomen. Induration and erythema were measured at 24 and 48 hr; a positive skin test was defined as greater than 5 mm induration. With this technique, 50% of nonimmune guinea pigs converted to a positive skin test indicating transfer of delayed hypersensitivity.

Experiments are now in progress to determine if passive transfer of serum or cells from immune to nonimmune guinea pigs will confer protection against challenge with virulent Q fever or VEE microorganisms.

Summary, Part II:

A technique for obtaining immunocompetent cells from the peritoneum of guinea pigs has been employed to transfer delayed hypersensitivity. Work is now in progress to assess the protective role of both hyperimmune sera and cells in infection of guinea pigs with VEE and Q fever.

Publications:

None.

LITERATURE CITED

1. Tomasi, Jr., T. B., and J. Bienenstock. 1968. Secretory immunoglobulins Adv. Immunology 9:1-96.

2. Sirisinha, S., and C. Charupatana. 1970. Antibody responses in serum secretions, and urine of man after parenteral administration of vaccines. Infect. Immun. 2:29-37.

3. Bellanti, J. A., E. L. Buescher, W. E. Brandt, H. G. Dangerfield, and D. Crozier. 1967. Characterization of human serum and nasal hemagglutinating antibody to Francisella tularensis. J. Immun. 98:171-178.

4. MacKaness, G. 1971. Resistance to intracellular infection. J. Infect. Dis. 123:439-445.

5. Blanden, R. V. 1970. Mechanisms of recovery from a generalized viral infection: Mousepox. I. The effects of anti-thymocyte serum. J. Exp. Med. 132:1035-1054.

ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 02:	Prevention and Treatment of Biological Agent Casualties
Work Unit No. 096 02 102:	Development and Evaluation of an Effective Vaccine Against Plague
Reporting Installation:	U.S. Army Medical Research Institute of Infectious Diseases Forest Glen Section Washington, D.C.
Division:	Microbiology
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Authors:	John D. Marshall, Jr., Colonel, MSC (II,III,IV) William H. Habig, Captain, MSC (I) Daniel N. Harrison, M.S. (III) Dan C. Cavanaugh, Lt Colonel, MSC (WRAIR) (II,III,IV) James H. Rust, Jr., Ph.D. (WRAIR) (II,III)
Reports Control Symbol:	RCS-MEDDH-288 (R1)
Security Classification:	UNCLASSIFIED

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA OL0833	71 07 01	DD DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 01 11	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	02	102
b. CONTRIBUTING				
c. ~~[illegible]~~	CDOG 1212b(9)			

11. TITLE (Precede with Security Classification Code)*
(U) Development and Evaluation of an Effective Vaccine against Plague (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*
003500 Clinical Medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
62 12	CONT	DA	C. In-house

17. CONTRACT GRANT
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER*:
c. TYPE: NA d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	3	85
	CURRENT 72	2	60

19. RESPONSIBLE DOD ORGANIZATION
NAME*: USA Medical Research Institute of Infectious Diseases
ADDRESS*: Fort Detrick, Md 21701
RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
NAME*: Microbiology Division USAMRIID
ADDRESS*: Fort Detrick, Md 21701
PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME*: Marshall, J. D.
TELEPHONE: 202 723-1000 Ext 5110
SOCIAL SECURITY ACCOUNT NUMBER:
ASSOCIATE INVESTIGATORS
NAME: Harrison, D. N.
NAME: DA

21. GENERAL USE
Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Pasteurella pestis; (U) Plague; (U) Vaccines; (U) Antigens; (U) Immunization; (U) Cytochrome; (U) Sentinal animals; (U) Military Medicine

23. TECHNICAL OBJECTIVE.* 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Determine the factors influencing the susceptibility to plague infection and the most appropriate method to prevent the infection.

24 (U) Using standard methods, sera from humans and animals are tested for the presence of fraction I antibody to P. pestis.

25 (U) 70 07 - 71 06 - The investigation of P. pestis cytochrome o has been completed. A report of this entire study will be submitted for journal publication shortly. Dogs, cats, and swine are susceptible to experimental plague infection; dogs and cats demonstrate P. pestis bacteremia as a result of the infection while swine do not. All 3 species examined respond to plague infection by developing antibodies to the specific fraction I antigen; which persist for at least 300 days in the sera of cats and dogs and 110 days in swine.

The antibody response of rhesus monkeys to immunization with killed plague vaccine was less uniform than with a living attenuated plague vaccine. All animals with demonstrable antibody at the time of challenge survived, while 2 of 3 without died of systemic plague.

In humans, there is little or no correlation between the quantity of plague vaccine administered and the serological response. After a finite number of booster inoculations are given, an antibody plateau level is reached in each individual. Additional immunization or its absence does not appear to alter markedly the antibody levels.

Publications: Bull. WHO 42:451-459, 1970; in Rapid Diagnostic Methods in Medical Microbiology, p. 67-81, 1970; Infect. Immun. 3:498-499, 1971. Infect. Immun. 1971, In press.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 096 02 102: Development and Evaluation of an Effective Vaccine Against Plague

Description:

Study the clinical and serological response of dogs, cats, monkeys, and swine to experimental plague infection. Determine the effectiveness of long term immunization in man.

Progress, Part I:

Studies on cytochrome o isolated from *Pasteurella pestis* have been completed.

The molecular weight and homogeneity of purified cytochrome o were investigated by discontinuous electrophoresis in various concentrations of polyacrylamide gel. The logarithm of the protein mobility was plotted against the acrylamide gel concentration. From the slope-molecular weight relationship determined with bovine serum albumin monomer, dimer, and trimer, the molecular weight of cytochrome o was estimated to be 160,000. This is in agreement with the previous estimate of 175,000 determined by gel filtration. In addition, the cytochrome o was homogeneous during electrophoresis over a wide range of gel concentrations.

Further studies in an Aminco-Chance dual wavelength spectrophotometer have substantiated initial observations that cytochrome o participates in the bacterial electron transport system between cytochrome b_{430} and molecular oxygen "Steady state" experiments were performed using washed *P. pestis* particles obtained by sonication. The degree of reduction of various components of the electron transport chain was examined spectrally in the presence of NADH or succinate. In both cases cytochrome o was about 6% less reduced than cytochrome b_{430}. Complete reduction with sodium dithionite indicated that about 20% of the cytochrome o does not participate in electron transport in such particles.

Summary, Part I:

The investigation of *P. pestis* cytochrome o has been completed. A report of this study will be submitted for journal publication shortly.

Progress, Part II:

Meyer[1/] (Medicine 21:143, 1942) postulated that dogs played an important role in the epidemiology of plague by conveying plague-infected fleas from field rodents to man. Blanc and Baltazard[2/] (Arch. Inst. Pasteur Maroc 3:175, 1945) isolated P. pestis from fleas collected from a cat and a dog found dead in a house where cases of plague had occurred. Pollitzer[3/] (Plague, WHO Monograph 22), in his review, stated that epizootics among the cat populations often coincided with epidemics of plague and Meyer[4/] (Med. Clin. N. Amer. 1943, p.756) was able to incriminate the cat as the source of infection for at least one case of plague.

Ten young, adult female beagles, 5 young, adult female cats, and 5 young, adult swine were utilized. The cats and dogs were infected by either the oral or parenteral routes, and the swine by the oral route only. Eight dogs and 3 cats were infected by the oral route. To simulate infection by fleas, 2 dogs and 2 cats were injected subcutaneously in the shaved scapular area with 0.2 ml of homogenate prepared in saline from the spleen of a guinea pig that had just died of plague. Sera obtained from the animals at varying intervals were examined for complement fixation (CF) and hemagglutinating (HA) antibodies to the fraction I antigen of P. pestis by the methods outlined by Cavanaugh et al [5/] (Bull. WHO 32:197, 1965).

Experimental plague infection in the dog -- Within 24-48 hr following infection, all 10 dogs showed some clinical signs of illness. While normally active and playful, the dogs became lethargic and unresponsive to handling. All dogs exhibited transient, mild to moderate, febrile response. The 2 most severely affected animals had a fever of 105 F for a period of 72 hr. By the 3rd day, fluctuant nonsuppurating abscesses had developed at the site of inoculation in both dogs which had received parenteral injections of infected material. P. pestis was repeatedly isolated from material aspirated from these lesions from days 3-10. Later attempts to isolate P. pestis were unsuccessful.

Plague bacilli were isolated sporadically from the throats of 4 of the 8 dogs infected by the oral route; through day 7 in 2 dogs; day 8 in 1 dog, and day 10 in 1 dog. The 2 dogs which displayed the most elevated temperatures were bacteremic; P. pestis was isolated from the blood of these 2 animals through day 3. Despite the fact that all of the dogs became ill, none of the animals succumbed to the disease and all appeared to have recovered clinically without treatment by day 7.

There was an abrupt appearance of both CF and HA antibody by day 8 and the peaks of HA antibody titer occurred between days 8 and 21. A decline from peak values to a constant HA antibody plateau was seen by day 100, and this level was maintained through at least day 315. By contrast, a steady decline in CF antibody occurred; this antibody was totally gone by day 200. There were no significant differences in the serological responses of dogs to P. pestis infection attributable to the route of administration.

Experimental plague infection in the cat -- Experimental plague infection appeared to be a very severe disease for the cat. All of the animals became acutely ill within 24-48 hr of infection. Uniformly, the animals demonstrated marked febrile responses, the temperatures rose from a normal of 101 F to a peak of 106 F. Two of the cats died during the period of acute illness on days 4 and 6. A 3rd cat which died as a result of the infection on day 20 had temperatures fluctuating between 103 F and 105 F throughout. The temperatures of the 2 surviving cats (1 oral and 1 parenterally infected) had returned to normal values by the 6th day.

P. pestis was isolated from the blood of all of the cats early in the course of disease and sporadically through day 20 in the cat with the protracted infection. From orally infected cats, P. pestis was isolated from the throats of 1 of 3 on day 3, 2 of 3 on day 6, and 1 of 3 on day 10. No isolations were obtained from the throats of animals infected by the parenteral route. One characteristic of feline plague was formation of large abscesses at the site of inoculation. Positive cultures were obtained from aspirates of these abscesses. Similar abscesses also appeared on the back, in the groin, and in the neck of one cat infected by the oral route. The neck abscess ruptured on day 9 and continued to discharge large quantities of purulent material from which P. pestis could be isolated until death on day 20.

When the 3 cats which died of plague were examined at autopsy, all animals had occasional discrete abscesses in spleen and liver. The lungs had similar large abscesses and evidence of extensive pneumonia. P. pestis was isolated in great numbers from the abscesses in all these organs and from the heart blood.

Due to the severity of the infection in the cat, cardiac puncture to obtain blood for serology was not attempted until day 12. At this time, HA antibody to the fraction I antigen was present in the sera of 2 of the cats at a titer of 1:2048 and 1:1024. By day 30, the HA titers in the sera of the 2 surviving cats had declined to a plateau of 1:512 which persisted through day 300. Cats did not produce CF antibody to fraction I antigen.

Experimental plague infection in swine -- Plague infection in the swine is a very mild disease. None of the animals had any signs of clinical illness; temperatures remained normal; and the animals remained active and alert. Repeated throat and blood cultures failed to yield P. pestis. All of the swine had HA antibody by day 7. Peak titers were reached by day 21 and declined to a plateau level of 1:1024-1:2048 by day 49 remaining constant through day 110. Swine do not produce CF antibody to fraction I antigen.

Summary, Part II:

Dogs, cats, and swine are susceptible to experimental plague infection. Both dogs and cats demonstrate P. pestis bacteremia as a result of the infection while swine do not. All 3 species studied respond to plague

infection by developing antibodies to the specific fraction I antigen. Such antibodies persist for at least 300 days in the sera of cats and dogs and 110 days in swine. All 3 species might serve as sentinel animals for the detection of plague activity in an area. Two papers have been prepared on dogs and cats.

Progress, Part III:

Serological response of rhesus monkeys to immunization and infection with P. pestis -- A commercial vaccine, plague, USP, E medium, Lot #K3400 (Cutter Laboratories), was used as the killed vaccine in this study. The fully competent living attenuated plague vaccine used was a freeze-dried strain of P. pestis EV76(51f), Lot 2. A recently passed strain of P. pestis 195/P with a mouse median lethal dose of <10 cells was used as the challenge organism.

Thirteen monkeys (Macaca mulatta) were assigned to 3 groups. Six animals were immunized intradermally (ID) with 1.0 ml Cutter vaccine on day 0 and given an 0.2 ml booster inoculation on day 90; 3 animals were inoculated intramuscularly (IM) with 10^9 viable EV76(51f) organisms on day 90; and 4 animals were fed 10^9 viable EV76(51f) in a banana on day 90. All animals were challenged ID on day 180 with 1×10^5 P. pestis 195/P.

Blood samples were collected at varying intervals. The blood was allowed to clot; the serum was removed aseptically. One ml of each serum was removed for immediate testing and the remainder was stored at -40 C for later testing. The microhemagglutination and microcomplement fixation tests of Cavanaugh, using purified fraction I antigen of P. pestis, were employed.

During the immunization phase of the study no adverse reactions, i.e., rise in temperature, change in behavior, regional lymphadenopathy or gastrointestinal disturbance were observed in any of the animals. Both the living and killed vaccines were well tolerated by the animals, regardless of the route of administration.

When challenged by the intradermal route with 2×10^5 P. pestis 195/P, all animals developed symptoms of infection, i.e., fever $\geq$104 F, loss of appetite, lethargy, and loss of condition within a period of 36 hr. Three animals, 2 of Group I and the control, died of septicemic plague on days 3 and 4. P. pestis was isolated from heart blood, peritoneal exudate, and thoracic fluid as well as from the cut surfaces of the lungs, spleen, and liver of all 3 monkeys. There was no evidence of regional lymphadenopathy. The remaining 10 monkeys developed eschar-like lesions at the site of inoculation with enlargement of the left femoral and inguinal lymph nodes. Material taken from the lesions yielded P. pestis on days 4-7 only. The lesions had healed and the lymph nodes had resolved by day 28 in all animals.

Primary inoculation of Group I with 1.0 ml killed vaccine failed to elicit demonstrable HA or CF antibodies. A second injection of 0.2 ml of this vaccine on day 90 resulted in the production of HA antibody by 3 animals and CF antibody by 2 animals by day 120. By contrast, all the animals receiving a single dose of living, attenuated P. pestis EV76(51f) vaccine by either the oral or parenteral routes responded by producing both HA and CF antibodies.

Following challenge, all animals that survived responded to the infection with a rapid rise in both HA and CF antibodies. Peak titers were observed between days 7-14 postinfection. During the next 200 days the titers, while declining somewhat from the values observed during the peak period, remained higher than those observed at any time during the immunization phase of the study.

It is noteworthy that 9 of 10 animals surviving infection with virulent P. pestis had demonstrable antibodies to fraction I antigen prior to infection. Two of 3 vaccinated animals in which antibodies could not be demonstrated succumbed to the disease within 4 days of challenge. The results of previous studies have demonstrated that unvaccinated control monkeys die in a 3-5 day period after challenge.

The results obtained when all of the sera which had been frozen at -40 C were tested simultaneously were identical to the results obtained when individual bleedings were tested prior to freezing.

Summary, Part III:

The antibody response of rhesus monkeys to immunization with killed plague vaccine was less uniform than with a living, attenuated plague vaccine. All animals having demonstrable antibody at the time of challenge survived, while 2 of 3 without demonstrable antibody died of systemic plague.

Progress, Part IV:

Long term multiple immunization of man with killed plague vaccine -- A series of 1,049 sera from 32 individuals receiving 5-34 plague immunizations were tested for HA and CF antibodies to the fraction I antigen of P. pestis by the methods of Cavanaugh et al[2].

The routine immunization procedures as applied to this group differed from those reported elsewhere in this report in that the sensitizing dose consisted of 3 injections of 0.5 ml, 1.0 ml, and 1.0 ml given at 7-day intervals followed by booster inoculation of either 0.5 ml or 0.25 ml every 6 mon as required. One or more preimmunization sera were available from 11 individuals. At least one serum taken after the primary series and after each of the first 2 booster inoculations were obtained. When the initial serological response of the 11 subjects who received an average of 3.0-3.5 ml of vaccine are compared with the volunteer group receiving a total of 1.4 ml of antigen, the results are quite similar. The positive geometric mean titers of the 2 groups differ by less than a factor of 2 as measured after the primary immunization, the 1st and 2nd booster inoculation.

When the 32 individuals are taken as a single group, several general conclusions can be drawn from the data: (1) After approximately 4 booster inoculations of killed plague vaccine, each individual reached a plateau with respect to HA antibody. Additional booster immunizations did not markedly

increase the plateau titer; (2) Once an individual reached a stabilized plateau, absence of further booster immunization for periods of 2-7 yr are reflected by either no loss or very gradual loss of HA antibody titers; (3) Both local and systemic reactions to plague vaccine tend to reoccur once an individual has experienced a single reaction. There does not appear to be either a qualitative or quantitative difference in the serological response in individuals who react adversely or do not react at all to plague vaccine; and, (4) There appears to be little or no correlation between the size of the dose, 0.1-0.5 ml, and HA response once an individual has received an adequate sensitizing dose of vaccine. Reduction of dose-size with or without medication does not reduce the chance of an adverse reaction in an individual with a history of previous reactions.

Summary, Part IV:

There is little or no correlation between the quantity of plague vaccine administered and the serological response. After a finite number of booster inoculations are administered, an antibody plateau level is reached in each individual. Additional immunization or its absence does not appear to markedly alter the antibody levels.

Publications:

1. Cavanaugh, D.C., P.J. Deoras, D.H. Hunter, J.D. Marshall, Jr., D.V. Quy, J.H. Rust, Jr., S. Purnaveja, and P.E. Winter. 1970. Some observations on the necessity for serological testing of rodent sera for *Pasteurella pestis* antibody in a plague control programme. Bull. WHO 42:451-459.

2. Wetzler, T.F., M.N. Rosen, and J.D. Marshall, Jr. 1970. The Pasteurella, the Yersinia and the Francisella, p.67-81. *In* C.D. Graber (ed.) Rapid Diagnostic Methods in Medical Microbiology, Williams and Wilkins Co., Baltimore.

3. Habig, W., B.W. Hudson, J.D. Marshall, Jr., D.C. Cavanaugh, and J.H. Rust, Jr. 1971. Evidence for molecular heterogeneity of the specific antigen (fraction I) of *Pasteurella pestis*. Infect. Immun. 3:498-499.

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1. Meyer, K.F. 1942. The ecology of plague. Medicine 21:143-174.

2. Blanc, G., and M. Baltazard. 1945. Recherches sur le mode de transmission naturelle de la peste bubonique et septicemique. Arch. Inst. Pasteur Maroc 3:175-354.

3. Pollitzer, R. 1954. Plague. World Health Organization Monograph 22. Geneva, Switzerland.

4. Meyer, K.F. 1943. Plague. Med. Clin. N. Amer., June, p.756.

5. Cavanaugh, D.C., B.D. Thorpe, J.B. Bushman, P.S. Cicholes, and J.H. Rust, Jr. 1965. Detection of an enzootic plague focus by serological methods. Bull. WHO 32:197-203.

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ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 02:	Prevention and Treatment of Biological Agent Casualties
Work Unit No. 096 02 300:	Immunologic Studies with Rickettsiae
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Division:	Virology
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Authors:	David M. Robinson, Major, VC (I) Richard H. Kenyon, Ph.D. (II)
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION: DA OL0885
2. DATE OF SUMMARY: 71 07 01
REPORT CONTROL SYMBOL: DD-DR&E(AR)636

3. DATE PREV SUM'RY: 71 01 06
4. KIND OF SUMMARY: D. CHANGE
5. SUMMARY SCTY: U
6. WORK SECURITY: U
7. REGRADING: NA
8a. DISB'N INSTR'N: NL
8b. SPECIFIC DATA-CONTRACTOR ACCESS: ☒ YES ☐ NO
9. LEVEL OF SUM: A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	02	300
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	CDOG 1212b(9)			

11. TITLE: (U) Immunologic Studies with Rickettsiae of Military Medical Importance (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical Medicine; 004900 Defense; 010100 Microbiology

13. START DATE: 67 07
14. ESTIMATED COMPLETION DATE: CONT
15. FUNDING AGENCY: DA
16. PERFORMANCE METHOD: C. In-house

17. CONTRACT/GRANT
a. DATES/EFFECTIVE:
EXPIRATION:
b. NUMBER:
c. TYPE: NA
d. AMOUNT:
e. KIND OF AWARD:
f. CUM. AMT:

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	2	40
	CURRENT 72	2	102

19. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, Md 21701
RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
NAME: Virology Division USAMRIID
ADDRESS: Fort Detrick, Md 21701
PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Robinson, D. M.
TELEPHONE: 301 663-4111 Ext 7241
SOCIAL SECURITY ACCOUNT NUMBER:
ASSOCIATE INVESTIGATORS
NAME: Kenyon, R. H.
NAME:
DA

21. GENERAL USE: Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Immunology; (U) Rickettsial diseases; (U) Spotted fevers; (U) Vaccines; (U) Q fever; (U) Coxiella burneti; (U) Military Medicine

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Develop vaccines of low reactogenicity for immunoprophylaxis against specific rickettsial diseases.

24 (U) Propagate representative strains in tissue culture systems. Assess the feasibility of producing rickettsial suspensions of quality and quantity suitable for vaccines for human use.

25 (U) 70 07 - 71 06 - I: The M-44 Strain of Coxiella burneti has been freed of rif viruses and redesignated the R-M (Rif -free M) strain. Master and working seeds have been produced and 5 lots of purified vaccine suitable for human use have been produced; testing is underway. Preliminary results indicate that certain primary cell cultures are good substrates for the growth of C. burneti.

II. A study of immunoprophylaxis against the spotted fever group was begun in April 1971. A duck-embryo cell grown, formalin-inactivated Rocky Mountain spotted fever (RMSF) vaccine had been developed elsewhere by the investigator. It appears to be superior to other available RMSF vaccines. Seed stocks of other members of the spotted fever group are available but probably contaminated with avian leukosis viruses. Attempts are in progress to remove the contaminant and proceed as with the RMSF product.
Publication: Bact. Proc., 1971, p. 85.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 — PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 096 02 300: Immunologic Studies with Rickettsiae

Description:

Develop vaccines of low reactogenicity for immunoprophylaxis against specific rickettsiae diseases.

Progress, Part I:

Research on the attenuated M Strain of *Coxiella burneti* during the last year has resulted in the production of 5 lots of yolk sac origin vaccine suitable for human use. The initial experiments were conducted to determine the response of M-44 experienced animals to challenge with phase I and II organisms. The phase I strain was the RIF-free (resistance inducing factor-free) vaccine (Henzerling origin) strain obtained from National Drug Co.; the phase II strain was Nine-Mile Strain (EP-88). The guinea pigs were inoculated with serial 10-fold dilutions of M-44 and held for 45 days. The challenge dose was standardized at 10,000 median lethal fever doses for guinea pigs (FD_{50}) for both strains. A decrease of 50% in the length of the febrile period in comparison to the controls was taken as index of protection. The dilution of M-44 which protected against the 2 challenge strains was the same with protection against EP-88 titering $10^{-8.3}$ and EP-3 titering $10^{-8.5}$.

The first requirement for a vaccine seed is freedom from detectable extraneous microorganisms. Since the M strain had been propagated in eggs not certified to be RIF-free, an aliquot of the WR-E1 material was heated at 56 C for 30 min to inactivate any viruses of this group which might be present. The 0.1-ml aliquot was immediately inoculated by the yolk sac route into certified RIF-free eggs (SPAFAS). On day 6 when 30% of the eggs alive on day 2 were dead the yolk sacs from the living eggs were harvested, processed into 20% suspensions in Snyder's I Buffer, labelled R-M (RIF-free M) Strain, E1 (first egg passage), and stored at -70 C. This represented master seed and an E2 passage was prepared as the working seed. Both of these were freed of rickettsia by egg passage in the presence of tetracycline and tested for RIF viruses at the Avian Viruses Unit, Departmentof Biologics Standards, National Institutes of Health; both were negative.

The R-M Strain E2 material was used to prepare a series of pools of infected yolk sacs for use in the development of a satisfactory purification procedure. All published techniques had been developed

to use with inactivated organisms and recovery rates had been followed by serological methods rather than measurement of infectivity. It was decided to approach the problem with both physical and chemical methods. The chemical method developed included a 30-min incubation at 37 C with a final concentration of 1.25% trypsin followed by low speed centrifugation. This procedure gave essentially 100% yields and was extremely simple and effective. (Median infective doses for eggs greater than 10^{12}/ml with nondialyzable N levels of <50 μg were common.) This method is used in the laboratory for the production of diagnostic antigens, but the use of trypsin was felt to be a dubious procedure in vaccine production for human use. Therefore, the final technique used for the purification of the vaccine involved 2 cycles of differential centrifugation followed by isopycnic density gradient centrifugation utilizing a 70% sucrose cushion. Nitrogen levels were elevated (approximately 600 μg N ml) and infectivity recovery rates were from 10-30%. Final titers were equal to or higher than those of the original yolk sac pools. The purified pools were sterility tested in fluid thioglycollate and Sabouraud's media, diluted to a standard concentration of $10^{9.5}$ ID_{50} for eggs per 0.1 ml, filled in 1-ml amounts in tubing glass vials, and freeze-dried at the Dept. Biologics Research, WRAIR.

Presently we are involved in safety, sterility, and potency testing of these 5 lots of freeze-dried attenuated Q Fever vaccine.

The search for an alternate system for rickettsial growth which will yield high titering material with low N levels has been initiated, and results with chick embryo fibroblast cell cultures are promising.

Summary, Part I:

C. burneti, M-44 strain has been freed of RIF-viruses and designated R-M strain. Master and working seeds were produced. Five lots of purified vaccine suitable for human use have been produced; testing is underway.

Progress, Part II:

There are at least 8 individually recognized diseases belonging to the spotted fever group of rickettsiae. Rocky Mountain spotted fever (RMSF) is recognized as the most severe and rickettsialpox, probably the mildest. All spotted fever diseases can be successfully treated with chloramphenicol or the tetracyclines; however, treatment with them presupposes that the physician is fully acquainted with the diseases and administers the appropriate treatment promptly. Due to the confusion in diagnosis and the severity of the spotted fever diseases the need for the availability of vaccines (monovalent or multivalent preparations) against the rickettsial agents is recognized.

To date, a vaccine has been produced for only one of the spotted fever group members, *Rickettsia rickettsii*, the causative agent of RMSF. The first vaccine for RMSF was a phenolized suspension of infected ticks[1]

(Public Health Rep. 40:2159, 1925). This was replaced by the Cox-type vaccine prepared from rickettsiae grown in fertile chicken egg yolk sacs, and killed with formaldehyde[2] (Public Health Rep. 54:1070, 1939). Accumulated experience indicates that these vaccines confer unsatisfactory levels of immunity in man[3,4] (JAMA 211:2012, 1970; Annual Progress Report, Contract DA 49-193-MD-2867, 15 Aug. 1970). Neither the yolk-sac grown vaccine nor the tick-grown vaccine is presently commercially available.

Immediate investigation is focused on the development of an effective vaccine for RMSF. A duck embryo cell (DEC) culture-grown, formaldehyde-inactivated vaccine has been developed and appears much superior to the yolk sac-grown vaccine. All seed material is almost certainly contaminated with avian leukosis viruses. Purification is being attempted by passing the seed material in duck yolk sacs, where leukosis virus replicates poorly or not at all, and then passage in chicken eggs free of avian leukosis virus. If this method is unsatisfactory physical methods of purification will be attempted. After a clean seed stock has been attained, a DEC-grown, formalin-inactivated vaccine will be prepared and its efficacy tested in guinea pigs, monkeys, and finally man.

Yolk-sac grown rickettsial suspensions of other members of the spotted fever group are available. They too most probably are contaminated with leukosis virus and purification is being attempted as with _R. rickettsii_. When working seeds have been attained and their parameters studied, cross protection studies will be initiated. Vaccines will be prepared for these other spotted fever group rickettsiae and their efficacy against homologous and heterologous challenge evaluated.

Summary, Part II:

A DEC-grown, formaldehyde-inactivated RMSF vaccine has been developed and appears considerably superior to the yolk sac-grown vaccine. Since all presently available spotted fever seed stocks are almost definitely contaminated with leukosis viruses, procedures are under way to purify these seed stocks.

Presentation:

1. Kenyon, R. H. Development of an improved vaccine for Rocky Mountain spotted fever. Presented at Annual Meeting of American Society for Microbiology, Minneapolis, Minn. 2-7 May 1971.

Publication:

1. Kenyon, R. H., W. M. Acree, F. W. Melchior, Jr., and G.G. Wright. 1971. Development of an improved vaccine for Rocky Mountain spotted fever. Bact. Proc. 1971, p. 85.

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1. Spencer, R. R., and R. R. Parker. 1925. Rocky Mountain spotted fever: vaccination of monkeys and man. Public Health Rep. 40:2159-2167.

2. Cox, H. R. 1939. Rocky Mountain spotted fever: Protective value for guinea pigs of vaccine prepared from rickettsiae cultivated in embryonic chick tissues. Public Health Rep. 54:1070-1077.

3. Calia, F. M., P. Bartelloni, and R. W. McKinney. 1970. Rocky Mountain spotted fever. Laboratory infection in a vaccinated individual. JAMA 211:2012-2013.

4. Hornick, R. B. 1970. Rocky Mountain spotted fever. In Annual Progress Report, 1 September 1969-15 August. Contract No. DA 49-193-MD-2867, University of Maryland School of Medicine.

ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 02:	Prevention and Treatment of Biological Agent Casualties
Work Unit No. 096 02 403:	Cross-Immunity Within the A Group of Arboviruses
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Division:	Virology
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Author:	Francis E. Cole, Jr. Ph.D.
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

331

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA OL0837	71 07 01	DD DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 01 06	H. TERMINATION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	02	403
b. CONTRIBUTING				
c. ~~CARDS 114f~~	CDOG 1212b(9)			

11. TITLE (Precede with Security Classification Code)*
(U) Cross-Immunity within the A Group of Arboviruses (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*
003500 Clinical Medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
61 08	71 06	DA	C. In-house

17. CONTRACT GRANT
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER*:
c. TYPE: NA d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	1	30
	CURRENT 72	0	0

19. RESPONSIBLE DOD ORGANIZATION
NAME:* USA Medical Research Institute of Infectious Diseases
ADDRESS:* Fort Detrick, Md 21701

RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
NAME:* Virology Division USAMRIID
ADDRESS:* Fort Detrick, Md 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME:* Cole, Jr., F. E.
TELEPHONE: 301 663-4111 Ext 7241
SOCIAL SECURITY ACCOUNT NUMBER

ASSOCIATE INVESTIGATORS
NAME: Hargett, H. H.
NAME: DA

21. GENERAL USE
Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Vaccines; (U) Arboviruses; (U) Immunization; (U) Encephalitis, equine (VEE, EEE, WEE)

23. TECHNICAL OBJECTIVE.* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Evaluate experimental attenuated and inactivated group A arbovirus vaccines for their ability to induce protection against other members of the group.

24 (U) Adult hamsters are inoculated with group A arbovirus vaccines in appropriate combinations and sequences. Response is determined by challenge with virulent strains and by serological techniques.

25 (U) 70 07 - 71 06 - All group A vaccines presently developed and tested have been examined for cross-protection against heterologous virus challenge. Except for the live, attenuated Venezuelan equine encephalomyelitis vaccine no heterologous protection was seen.

This work unit has been terminated. Related studies will be published under Work Unit No. 096 03 407 in the future.

Publication: Infect. Immun., 1971, In press.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 096 02 403: Cross-immunity Within the A Group of Arboviruses

Description:

Evaluate experimental attenuated and inactivated group A arbovirus vaccines for their ability to induce protection against other members of the group.

Progress and Summary:

All group A vaccines presently developed and tested have been examined for cross-protection against heterologous virus challenge. Except for the live, attenuated Venezuelan equine encephalomyelitis vaccine no heterologous protection was seen.

This work unit has been terminated. Related studies will be published under Work Unit No. 096 03 407 in the future.

Publications:

1. Cole, F. E., Jr., and R. W. McKinney. 1971. Cross-protection in hamsters immunized with group A arbovirus vaccines. Infect. Immun., In press.

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ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 02:	Prevention and Treatment of Biological Agent Casualties
Work Unit No. 096 02 407:	Development of Inactivated Arbovirus Vaccines
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Division:	Virology
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Authors:	Francis E. Cole, Jr., Ph.D.
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSFIED

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
	DA OL0841	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 03 08	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	02	407
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1212b(9)			

11. TITLE (Precede with Security Classification Code)
(U) Development of Inactivated Arbovirus Vaccines for Diseases of Military Importance (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
003500 Clinical Medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
64 06	CONT	DA	C. In-house

17. CONTRACT GRANT
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER:
c. TYPE: NA d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	1	50
	CURRENT 72	1	66

19. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, Md 21701
RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
NAME: Virology Division USAMRIID
ADDRESS: Fort Detrick, Md 21701
PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Cole, Jr., F. E.
TELEPHONE: 301 663-4111 Ext 7241
SOCIAL SECURITY ACCOUNT NUMBER
ASSOCIATE INVESTIGATORS
NAME:
NAME:
DA

21. GENERAL USE
Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Vaccines; (U) Encephalitis, equine (EEE, VEE); (U) Mayaro; (U) O'Nyong-nyong; (U) St Louis encephalitis; (U) California encephalitis; (U) Cell culture (U) Milit. Med.

23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Produce inactivated arbovirus vaccines which may be combined selectively for prophylaxis in specific geographically oriented ways.

24 (U) Arboviruses are propagated in primary cell culture and inactivated with formalin. Products are tested for safety and potency in animals. Efficacy is determined by subsequent challenge, or by determination of serological conversion.

25 (U) 70 07 - 71 06 - Eighteen small lots of potent EEE vaccine have been prepared in roller bottle cultures of chick embryo cells (CEC). The following parameters for EEE vaccine production are presented: (1) a multiplicity of inoculum (MOI) of 0.005 may be employed yielding maximum titers of virus 18-24 hr postinoculation; (2) less than 300 ml of maintenance medium may be employed without decreasing final potency; and (3) inactivation by USPHS standards at 37 C may be carried out with 0.05% formalin for 48 hr or 0.1% formalin for 24 hr.

Utilizing similar methods 30 small lots of potent VEE vaccine have been prepared. The following parameters for production are presented: (1) using a MOI of 0.006, CEC cultures yield maximum titers of virus at 20-24 hr postinoculation; (2) less than 300 ml of maintenance medium may be used without adversely affecting potency; and (3) inactivation by USPHS standards at 37 C may be carried out with 0.05% or 0.1% formalin for less than 24 hr, although formalin treatment may be extended for 96 hr without apparent deleterious effects on potency.

Studies have recently been initiated toward the concurrent development of inactivated vaccines for Mayaro, O'Nyong-nyong and California and St. Louis encephalitis.

Publication: Amer. J. Trop. Med. Hyg. 20:146-149, 1971.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 096 02 407: Development of Inactivated Arbovirus Vaccines

Description:

Produce inactivated arbovirus vaccines which may be combined selectively for prophylaxis in specific geographically oriented ways.

Progress:

Using previously described[1/] (USAMRIID Annual Progress Report, FY 1969, p. 157) methods, small lots of formalin-inactivated Eastern equine encephalitis (EEE), and Venezuelan equine encephalomyelitis (VEE) vaccines have been produced. In addition studies have recently been initiated to develop inactivated vaccines for Mayaro (MAY), O'nyong-nyong (ONY), California encephalitis (CAL), and St. Louis encephalitis (SLE).

EEE virus, PE-6 strain, was obtained from Walter Reed Army Institute of Research as an egg suspension with a history of 13 intracerebral (IC) mouse passages, followed by 6 passages in embryonated hens' eggs. The virus was subjected to 2 additional passages in embryonated eggs at USAMRIID, with the virus from the 2nd egg passage serving as seed for vaccine production and challenge virus for potency assays.

Trinidad strain VEE virus had a history of 1 guinea pig passage followed by 13 passages in embryonated hens' eggs. This virus was passaged in rolling bottle cultures of chick embryo cell (CEC) cultures to produce seed for vaccine production.

Both the EEE and VEE viruses were titrated using 3-week-old white mice (CD-1 strain of Charles River Mouse Farms, Wilmington, Mass.). Virus samples were diluted in cold phosphate buffered saline containing 1% normal rabbit serum. Groups of 5 mice were inoculated intracerebrally (IC) with 0.03 ml of $\log_{10}$ dilutions of virus-containing fluids and were observed for 10 days for deaths. Titration endpoints were determined by the method of Reed and Muench[2/] (Amer. J. Hyg. 27:493, 1938) and expressed as median lethal doses (LD_{50}) per 1.0 ml.

Nine-day-old chick embryos were minced and trypsinized according to conventional methods. The resulting cell preparations were suspended to a final concentration of 4 X 10^6 cells/ml in a growth medium consisting of Eagle's basal medium containing 10% calf serum and 1% glutamine, plus 100 µg/ml each of neomycin, U.S.P. and streptomycin, U.S.P. Bellco 840-cm^2 cell production roller vessels (Bellco Glass, Inc., Vineland, N. J.) were seeded with 170-ml portions of the cell suspension, placed on a Bellco roller apparatus, and incubated at 35 C until confluent cell sheets were obtained (18-24 hr). Once all confluency was achieved, the growth medium was decanted and replaced with serum-free medium 199 containing neomycin and streptomycin as in the growth medium. The CEC roller cultures were held an additional 20-24 hr at 35 C. Prior to infection, the medium was removed and the residual fluid was drained from the cultures.

Potency assays of EEE vaccines was carried out in Lakeview strain Golden Syrian hamsters (85-95 gm) (Lakeview Hamster Colony, Newfield, N. J.). Groups of 10 hamsters were inoculated intraperitoneally (IP) on days 0 and 7 with 0.5 ml of 5-fold dilutions of vaccine. Challenges with virulent EEE virus were performed 21 days after the last vaccine dose using 10^3 - 10^4 hamster $IPLD_{50}$. Animals were observed for deaths for 14 days. Titration endpoints were determined by the method of Reed and Muench.[2] The potency of a vaccine was expressed as the median effective dose (ED_{50}), i.e. the volume of undiluted vaccine given in each dose of the 2-dose series which protected 50% of the hamsters from death following challenge.

VEE vaccines were assayed using 3-week-old CD-1 white mice. Groups of 10 mice were inoculated IP on day 0 with 0.3 ml of 5-fold dilutions of vaccine. Fourteen days later the mice were challenged IP with 10^3 - 10^4 mouse $IPLD_{50}$ of virulent VEE. Titration endpoints and ED_{50} values were determined as described for EEE vaccines.

Roller bottle CEC cultures were infected with PE-6 strain EEE virus at multiplicities of inoculum (MOI) of 5.0 - 0.0005 and maintained with 300 ml of medium 199 containing neomycin, streptomycin and 0.25% human serum albumin. Samples of culture fluids were removed at regular intervals postinoculation and assayed in mice. As shown in Table I, optimum yields of virus were obtained at a convenient harvest time of 18-24 hr at an acceptable MOI of 0.005.

TABLE I. EFFECT OF MOI ON PROPAGATION OF EEE VIRUS IN ROLLER BOTTLE CEC CULTURES

HOURS POST-INOCULATION	LOG_{10} LD_{50}/ML BY MOI				
	5.0	0.5	0.05	0.005	0.0005
6	≧7.0	≧6.7	≧7.0	6.5	5.0
12	≧8.9	>9.0	≧9.0	>8.3	≧8.7
18	9.2	9.0	8.8	8.7	8.7
24	9.0	9.0	9.1	9.2	8.2
30	8.5	8.0	8.7	9.0	≦8.5

Since previous experience in this laboratory suggested that EEE virus yield may be affected by maintenance medium volume, replicate cultures were infected at an MOI of 0.005 and then maintained with 100 - 300 ml of medium 199. The results of titrations performed with fluids removed from these cultures at regular intervals are shown in Table II. Although use of 100 or 200 ml of maintenance medium resulted in somewhat higher yields of virus at a convenient harvest time (18-24 hr), the cultures thus maintained showed far greater cytopathic effects (CPE) than those maintained with 300 ml.

TABLE II. EFFECT OF MAINTENANCE MEDIUM VOLUME ON PROPAGATION OF EEE VIRUS IN CEC ROLLER BOTTLE CULTURES (MOI = 0.005)

HOURS POST-INOCULATION	LOG_{10} LD_{50}/ML BY MAINTENANCE MEDIUM VOLUME		
	100 ml	200 ml	300 ml
6	7.2	7.0	6.8
12	9.9	9.1	9.1
18	10.0	9.5	9.1
24	10.3	9.8	9.3
30	9.3	9.3	9.3

Thus, the amount of total protein nitrogen can be expected to be greater in the 100- or 200-ml harvests. Assays of additional 18- and 24-hr harvests from cultures maintained with 100-300 ml of medium indicate that significantly higher titers are not consistently obtained with the smaller volumes of medium. In all cases, however, titers of ≥ $10^{9.0}$ LD_{50}/ml were obtained regardless of maintenance medium volume.

For final processing into vaccines, all harvests were clarified by centrifugation at 900 g for 30 min at 4 C, followed by filtration through a 0.45µ membrane filter (Millipore). These steps ensured removal of cellular debris which might adversely affect virus inactivation. Studies at USAMRIID (unpublished data) have indicated that little or no loss of infectivity occurs as a result of these clarification measures.

Inactivation of clarified virus harvests was accomplished by addition of formalin (Formaldehyde, 37% assay) to a final concentration of 0.05 or 0.1%. After addition of formalin the fluids were thoroughly mixed by shaking and incubated at 37 C for 24 to 48 hr. During this period the flasks were agitated frequently. At the end of the desired period of inactivation, the material was held at 4 C for 15 days, during which time the vessels were shaken at least twice daily.

Samples of vaccine taken at regular intervals during the inactivation period were titrated in mice; samples of harvests subjected only to 37 C for similar periods were included as controls. Shown in Table III are representative results of such titrations which have been performed on the majority of vaccines thus produced.

With 0.05% formalin "complete inactivation" generally occurred in 16 hr; using a final concentration of 0.1%, "complete inactivation" occurred in 8 hr. The exact "killing time" varied slightly from lot to lot and therefore must ve determined for each individual lot. Since 1970 U. S. Public Health Service standards, Title 42, Part 73 require formalin treatment at 37 C for 3 times that period required for "complete inactivation," this would entail a 48 hr inactivation period for 0.05% vaccines and a 24 hr period for the 0.1% vaccines.

TABLE III. FORMALIN INACTIVATION[a] OF EEE VIRUS AT 37 C

HOUR POST-FORMALIN	LOG_{10} LD_{50}/0.03 ML BY FORMALIN CONCENTRATION 0.05%	0.1%	VIRUS[b] CONTROL
0	7.7 - 8.7[c]	7.7	7.7
2	2.9 - 4.4	1.6	-
4	1.3 - 1.8	1.0	-
6	<1.0	1.0	-
8	<1.0	0[d]	4.0
10	<1.0	0	-
12	<1.0	0	-
16	0	0	-
18	0	0	-
24	0	0	-
30	0		<2.0

a. Determined by IC inoculation of adult mice with 0.03 ml.

b. - = Not tested.

c. Range observed on samples from different lots of vaccine.

d. 0 = survival of 50/50 mice inoculated with undiluted vaccine.

Both extended contact with formalin (particularly in higher concentrations) and exposure to temperatures above 0 C may result in decreased antigenicity in vaccines, and thus, lower potencies. To evaluate the effect of these factors on EEE vaccines, 18 small lots of experimental vaccines were prepared using either 0.05% or 1.0% formalin and inactivation periods of 24 - 48 hr. Shown in Table IV are the results of potency assays performed on vaccines prepared during this study.

TABLE IV. EFFECT OF FORMALIN CONCENTRATION AND LENGTH OF INACTIVATION PERIOD ON POTENCY OF EEE VACCINES

FORMALIN CONCENTRATION %	ED_{50} (ML) BY HOURS OF INACTIVATION (range) 24	30	36	48
0.05	0.035[a] (0.028-0.045)	0.030[b] (0.012-0.069)	0.025[c] (0.020-0.034)	0.015
0.1	0.023	0.033	0.030	-

a. Mean of 3 lots.

b. Mean of 7 lots.

c. Mean of 4 lots.

These results suggest that potent EEE vaccines may be produced with either 0.05% formalin using a long (e.g., 48 hr) inactivation period or with 0.1% formalin using shorter periods of inactivation. Not shown in Table IV are the volumes of maintenance medium employed on cultures used to produce these vaccines, and the titers of the viral harvests prior to final processing. Maintenance medium volumes ranged from 100-300 ml, while preinactivation titers varied from $10^{9.3}$ to $10^{10.2}$. No correlation could be made between preinactivation titers and vaccine potencies. Apparently even the lowest titers achieved represented sufficient antigenic mass for potent vaccines. It, therefore, appears feasible to use 300 ml of maintenance medium for high volume production without a concomitant decrease in potency of the final product.

Using methods described for EEE virus, 30 laboratory scale lots of inactivated VEE vaccine have been produced and assayed for potency.

Roller bottle cultures were infected at MOI of 0.06 - 0.00006 and maintained with 200 ml of medium 199. Culture fluid samples removed from these bottles at 20 and 24 hr postinoculation were titrated in mice. As indicated in Table V, good virus yields were obtained at all MOI levels. In all further studies reported here, an MOI of 0.006 was employed. This represents a 10^{-4} dilution of the CEC virus seed material.

TABLE V. EFFECT OF MOI ON PROPAGATION OF VEE VIRUS IN ROLLER BOTTLE CEC CULTURES

HOURS POST-INOCULATION	LOG_{10} LD_{50}/ML BY MOI			
	0.06	0.006	0.0006	0.00006
20	8.5	8.9	9.0	8.5
24	8.7	8.3	8.5	9.0

To determine the effect of maintenance medium volume on virus yield, CEC cultures were inoculated as previously described, and maintained with 100, 200 or 300 ml of medium 199 with neomycin, strepytomycin and human serum albumin. Summarized in Table VI are the results of assays performed on fluids removed from these cultures at various periods postinoculation. Although adequate virus titers were achieved at all volumes between 12 and 18 hr postinoculation, from a production standpoint the larger volumes

would be most advantageous. Cell destruction was moderate at 12 hr but increased greatly by 24 hr postinoculation. Thus, high titered virus material with lesser amounts of cellular debris could best be obtained with a harvest at 18 - 20 hr.

TABLE VI. EFFECT OF MAINTENANCE MEDIUM VOLUME ON PROPAGATION OF VEE VIRUS IN CEC ROLLER BOTTLE CULTURES (MOI = 0.000)

HOURS POST-INOCULATION	LOG_{10} LD_{50}/ML[a] BY MAINTENANCE MEDIUM VOLUME		
	100 ml	200 ml	300 ml
2	3.2	3.3	3.0
6	6.5	6.3	6.0
12	9.9	9.0	9.0
18	9.9	10.3	9.9
24	10.0	9.3	9.3

a. Mean titers of samples o tained from replicate sets of cultures.

Thirty small lots of VEE vaccine have been prepared using procedures described for EEE with regard to clarification and addition of formalin to final concentrations of 0.05 and 0.1%. However, inactivation was performed at 37 C in flasks placed in a New Brunswick Reciprocating Water Bath. The fluids were thus kept in gentle but constant movement throughout the inactivation periods of 24 - 96 hr. Typical rates of formalin-inactivation are shown in Table VII. Suprisingly VEE succumbed at a rapid rate with both 0.05% and 0.1% formalin, but was quite stable when subjected to heat only (virus control).

TABLE VII. FORMALIN INACTIVATION[a] OF VEE VIRUS AT 37 C

HOURS POST-FORMALIN	LOG_{10} LD_{50}/0.03 ML BY FORMALIN CONCENTRATION		VIRUS[b] CONTROL
	0.05%	0.1%	
0	10.0	10.0	10.0
2	3.3	3.0	-[c]
4-16	0[d]	0	-
24	0	0	8.7
96	-	-	3.0

a. Determined by IC inoculation of adult mice with 0.03 ml.

b. Virus subjected to 37 C only.

c. - not tested.

d. 0 = survival of 50/50 mice inoculated at 8-16 hr with undiluted vaccine.

As with EEE, VEE vaccines were inactivated for extended periods to determine the effect on potency. Table VIII is a summary of the results of single-dose potency assays performed on 30 lots of VEE vaccine prepared with 0.05% and 0.1% formalin and inactivated for 24 - 96 hr. These data indicate that VEE is quite stable, antigenically, even after treatment with formalin for 96 hr. The volume of maintenance medium and pre-inactivation titers of the fluids used to prepare these vaccines varied from 100 - 300 ml and $10^{8.3}$ to $10^{10.7}$ $MICLD_{50}$/ml respectively. There is no apparent correlation between these values and the potencies observed. One can only conclude that formalin-inactivated VEE vaccine may be made with larger volumes of maintenance medium and with great latitude with regard to period of inactivation.

TABLE VIII. EFFECT OF FORMALIN CONCENTRATION AND LENGTH OF INACTIVATION PERIOD ON POTENCY OF VEE VACCINES

FORMALIN CONCENTRATION -10	ED_{50} (ML) BY HOURS OF INACTIVATION (range) 24	30	48	72	96
0.05	0.032[a]	0.040[b]	0.032	0.021	0.012[c]
	(0.026-0.042)	(0.0072-0.11)			(0.0041-0.019)
0.1	0.015	0.017[d]	0.026	0.0081	0.014[c]
		(0.0039-0.030)			(0.0054-0.034)

a. Mean of 3 lots.

b. Mean of 12 lots.

c. Mean of 4 lots.

d. Mean of 2 lots.

In preparation for the concurrent development of inactivated vaccines for Mayaro (MAY), O'nyong-nyong (ONY), California encephalitis (CAL), and St. Louis encephalitis (SLE), permits were secured for the shipment of available strains from the American Type Culture Collection, Rockville, Md. The Osege strain of ONY, unavailable elsewhere, was obtained from Dr. Allen of the Virus and Rickettsia Division, Biological Defense Research Center. All viruses were received as suckling mouse brain (SMB) preparations. Master and working SMB seeds have been prepared for all 4 viruses in mice obtained from the Fort Detrick Animal Farm Division.

To provide baseline data on the growth of these viruses in cell culture, the following primary cell cultures were inoculated with a 10^{-3} dilution, (nb., empirical, since no hard data is available) of a given virus: canine kidney, rabbit kidney, hamster kidney, chick embryo, duck embryo, and monkey kidney, African Green; the human diploid cell line, WI-38 was also studied. After inoculation all cell cultures were maintained on medium 199 containing 0.25% human serum albumin, and 50 μg/ml each of penicillin and streptomycin. Fluid samples were taken from infected cultures at 18 and 24 hr postinoculation and frozen until titrated in suckling mice; the degree of cell destruction was recorded at the time of sampling.

Since USPHS Regulations require that foreign protein, if present, must not exceed a concentration of 1:1,000,000, it was necessary to ascertain whether serial subculture of the 4 viruses could be made in selected cell cultures; such serial passage, if possible without a significant decrease in the titer of the viruses, would obviously reduce the level of mouse brain antigen (source of the original viruses) to an insignificant level. Thus all viruses are being serially passed in those cell cultures in which cell destruction was greatest. At the time of writing the 4 viruses had been passed 3 times in both primary hamster kidney and duck embryo, and had been passed 2 times in primary canine kidney and African green monkey kidney cells.

Titrations of cell culture fluid samples (i.e., 18 and 24 hr) are in progress utilizing IC inoculation of suckling mice, the adult mouse having been shown to be resistant to, or irregularly susceptible to the 4 viruses under study.

Summary:

Eighteen small lots of potent EEE vaccine have been prepared in roller bottle cultures of chick embryo cells (CEC). The following parameters for EEE vaccine production are presented: (1) a multiplicity of inoculum (MOI) of 0.005 may be employed yielding maximum titers of virus 18-24 hr postinoculation; (2) ' 300 ml of maintenance medium may be employed without decreasing final potency; and (3) inactivation by USPHS standards at 37 C may be carried out with 0.05% formalin for

48 hr or 0.1% formalin for 24 hr.

Utilizing similar methods 30 small lots of potent VEE vaccine have been prepared. The following parameters for production are presented: (1) using a MOI of 0.006, CEC cultures yield maximum titers of virus at 20 0 24 hr postinoculation; (2) ≤ 300 ml of maintenance medium may be used without adversely affecting potency; and (3) inactivation by USPHS standards at 37 C may be carried out with 0.05% or 0.1% formalin for ≤ 24 hr, although formalin treatment may be extended for 96 hr without apparent deleterious effects on potency.

Studies have recently been initiated toward the concurrent development of inactivated vaccines for Mayaro, O'nyong-nyong and California and St. Louis encephalitis.

Publications:

1. Bartelloni, P. J., R. W. McKinney, F. M. Calia, H. H. Ramsburg, and F. E. Cole, Jr. 1971. Inactivated Western equine encephalomyelitis vaccine propagated in chick embryo cell culture. Clinical and serological evaluation in man. Amer. J. Trop. Med. Hyg. 20:146-149.

LITERATURE CITED

1. U. S. Army Medical Research Institute of Infectious Diseases. 1 July 1969. Annual progress report, FY 1969, p. 157 to 166. Fort Detrick, Maryland

2. Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty per cent endpoints. Amer. J. Hyg. 27:493-497.

ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 02:	Prevention and Treatment of Biological Agent Casualties
Work Unit No. 096 02 408:	Role of Antibody in the Clinical Manifestations of Venezuelan Equine Encephalomyelitis
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Division:	Medical
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Authors:	Peter J. Bartelloni, Lt Colonel, MC Nemesio M. Francisco, Major, MC
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

236

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
	DA OL0811	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 01 06	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	02	408
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	CDOG 1212b(9)			

11. TITLE (Precede with Security Classification Code)*
(U) Role of Antibody in the Clinical Manifestations of Venezuelan Equine Encephalomyelitis (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*
003500 Clinical Medicine; 004900 Defense; 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
62 02	CONT	DA	C. In-house

17. CONTRACT/GRANT
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER:*
c. TYPE: NA d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	0	10
	CURRENT 72	1	6

19. RESPONSIBLE DOD ORGANIZATION
NAME:* USA Medical Research Institute of Infectious Diseases
ADDRESS:* Fort Detrick, Md 21701
RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
NAME:* Medical Division USAMRIID
ADDRESS:* Fort Detrick, Md 21701
PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME:* Bartelloni, P. J.
TELEPHONE: 301 663-4111 Ext 6135
SOCIAL SECURITY ACCOUNT NUMBER:
ASSOCIATE INVESTIGATORS
NAME: Francisco, N. M.
NAME:
DA

21. GENERAL USE
Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Military Medicine; (U) Prophylaxis; (U) Encephalitis, equine (VEE); (U) Virus diseases; (U) Immune serum

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Study the role of antibody as it relates to the clinical manifestations of Venezuelan equine encephalomyelitis (VEE) virus infection, and the usefulness of immune serum in prophylaxis and treatment of this infection.

24 (U) Animals are inoculated with either attenuated or virulent VEE virus. The efficacy of antiserum in preventing undesirable reactions to these viruses is evaluated. The resulting immune response and its dependency on the relationship of the quantity of antiserum given to time of its administration is investigated.

25 (U) 70 07 - 71 06 - During the year, no tests were performed. This is a work unit needed for future work as required.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 096 02 408: Role of Antibody in the Clinical Manifestations of Venezuelan Equine Encephalomyelitis

Description:

Study the role of antibody as it relates to the clinical manifestations of Venezuelan equine encephalomyelitis (VEE) virus infection, and the usefulness of immune serum in prophylaxis and treatment of this infection.

Progress and Summary:

During the year, no tests were performed.

Publications:

None.

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ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 02:	Prevention and Treatment of Biological Agent Casualties
Work Unit No. 096 02 410:	Pathophysiology, Pathogenesis and Therapy of Yellow Fever
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Divisions:	Animal Assessment and Pathology
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Authors:	Richard O. Spertzel, Lt Colonel, VC (I) James L. Stookey, Lt Colonel, VC (I) Philip C. Kosch, Captain, VC (I) Steven H. Gilbertson, Captain, VC (II)
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

240

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
	DA OL0877	71 07 01	DD DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 02 09	D. CHANGE	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	02	410
b. CONTRIBUTING				
c. ~~CARDS 114~~	CDOG 1212b(9)			

11. TITLE (Precede with Security Classification Code)
(U) Pathophysiology, pathogenesis and therapy of yellow fever (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
003500 Clinical Medicine; 004900 Defense; 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
66 12	CONT	DA	C. In-house

17. CONTRACT GRANT
a. DATES/EFFECTIVE — EXPIRATION
b. NUMBER
c. TYPE NA — d. AMOUNT
e. KIND OF AWARD — f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	2	45
	CURRENT 72	2	59

19. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, Md 21701
RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
NAME: Animal Assessment and Pathology Div USAMRIID
ADDRESS: Fort Detrick, Md 21701
PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Spertzel, R. O.
TELEPHONE: 301 663-4111 Ext 7244
SOCIAL SECURITY ACCOUNT NUMBER
ASSOCIATE INVESTIGATORS
NAME: Kosch, P. C.
NAME: Gilbertson, S. H. DA

21. GENERAL USE
Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Yellow fever; (U) Pathology; (U) Therapy; (U) Physiology, medical; (U) Military medicine

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) A multidisciplinary approach is used for the study of the pathogenesis and pathophysiology of yellow fever.

24 (U) Inoculate laboratory animals with yellow fever virus, Asibi and 17-D vaccine strains. Study changes in blood and tissues and alterations in organ function during the course of the disease. Such fundamental knowledge is needed prior to testing methods of prevention and treatment.

25 (U) 70 07 - 71 06 - I: Existing data on the effects of yellow fever on rhesus monkeys are being collected as background. Experimental work will be initiated in the near future.

II: Fundamental information on ultrastructural changes resulting from yellow fever infection is needed. A preliminary study in mice using the 17-D vaccine strain was recently started. Eventually, larger laboratory animals will be similarly examined.

Publication: Lab. Anim. Sci., 1971, In press.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 096 02 410: Pathophysiology, Pathogenesis and Therapy of Yellow Fever

Description:

A multidisciplinary approach is used for the study of the pathogenesis and pathophysiology of yellow fever.

Progress and Summary, Part I:

Yellow fever infection in the rhesus monkey will be used as an experimental model to study various parameters, e.g., trace metals, free amino acids, renal and hepatic function, pathology. In support of and correlated with these studies, a set of standardized clinical and laboratory test evaluations will be performed on each infected animal in order to adequately relate the various findings to the infectious model.

Initially, studies relative to the early onset of clinical symptoms and relationship of inoculum dose to incubation period will be collated and augmented. In association with Physical Sciences and Pathology Divisions, pilot studies relative to the various parameters to be measured will be conducted to determine optimum utilization of time, space and personnel.

Studies have just been initiated and no progress to be reported.

Progress and Summary, Part II:

There have been few electron microscopic studies of yellow fever virus; results have been inconsistent and conflicting. The size and structure of the virus and the ultrastructural changes it induces have not been well characterized. It is the purpose of this project to detail the ultrastructural pathology induced by yellow fever virus. 17-D vaccine strain virus will be inoculated into mouse brains and tissue cultures which will then be examined by electron microscopy, immunofluorescence, and light microscopy in conjunction with viral assay of the same tissues. The 17-D strain itself will be purified from the vaccine by ultracentrifugation and examined under the electron microscope to establish its size and structure.

Publications:

Chapple, F. E., III, J. M. Crosbie, and B. E. Reisberg. 1971. Surgical Technique for cross-circulation of rhesus monkeys. Lab. Anml. Sci. (In press).

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ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 02:	Prevention and Treatment of Biological Agent Casualties
Work Unit No. 096 02 411:	Evaluation of Promising Compounds for Antiviral Use
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Division:	Animal Assessment
Period Covered by Report:	22 December 1970 to 30 June 1971
Professional Author:	Donald E. Kahn, Captain, VC
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION: DA OL0907	2. DATE OF SUMMARY: 71 07 01	REPORT CONTROL SYMBOL: DD DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
70 12 22	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	02	411
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	CDOC 12126(9)			

11. TITLE (Precede with Security Classification Code): (U) Evaluation of Promising Compounds for Antiviral Use against Diseases of Medical Importance to the Military (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 010100 Microbiology; 003500 Clinical Medicine; 004900 Defense; 001700 Animal Husbandry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
70 12	CONT	DA	C. In-house

17. CONTRACT/GRANT: a. DATES/EFFECTIVE: EXPIRATION: b. NUMBER: c. TYPE: NA d. AMOUNT: e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	1.5	10
	CURRENT 72	1	62

19. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, Md 21701
RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
NAME: Animal Assessment Division USAMRIID
ADDRESS: Fort Detrick, Md 21701
PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Spertzel, R. O.
TELEPHONE: 301 663-4111 Ext 7244
SOCIAL SECURITY ACCOUNT NUMBER
ASSOCIATE INVESTIGATORS
NAME: Kahn, D. E.
NAME:
DA

21. GENERAL USE: Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Military Medicine; (U) Therapy; (U) Virus diseases; (U) Tissue culture; (U) Laboratory animals

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Evaluate chemical compounds for treatment and control of virus diseases of importance to the military.

24 (U) Test chemicals in tissue culture and laboratory animals against selected viruses.

25 (U) 70 12 - 71 06 - Two model systems were examined preparatory to testing antiviral agents. In the first, an inverse relationship between dosage of virulent Asibi strain yellow fever virus and duration of clinical illness was observed in rhesus monkeys. The incubation period did not change with virus dose. Thus, at the lower doses illness was prolonged sufficiently to permit testing of treatments.

In the second model of VEE in mice, no evidence of protection was observed when splenic heterotransplants to mice were made before infection with the virus.

Methods are being developed to perform plaque-reduction, serum-neutralization testing using microtitration equipment.

*Available to contractors upon originator's approval

DD FORM 1498, 1 MAR 68 — PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 096 02 411: Evaluation of Promising Compounds for Antiviral Use

Description:

Identification of compounds with therapeutic or prophylactic use in virus infections.

Progress:

During the past year, a research plan for the evaluation of antiviral compounds was written and approved. Work has begun in the development of 2 model systems of infection for use in the program, yellow fever in monkeys and Venezuelan equine encephalomyelitis (VEE) in mice.

In an attempt to investigate the role of dose yellow fever response, rhesus monkeys (*Macaca mulatta*) were inoculated subcutaneously with Asibi strain virus in dosages ranging from 5-1,000 median mouse intracerebral lethal doses ($MICLD_{50}$).

Of the 12 monkeys used in this trial, one survived the acute infection. This monkey (A-256) responded to inoculation with 100 $MICLD_{50}$ of virus with a body temperature of $\geq$ 103 F for 76 hr. The febrile response commenced 72 hr postinoculation. A maximum body temperature of 105.4 F was recorded at 120 and 132 hr postinoculation. The monkey exhibited leukopenia, lymphopenia, and depressed hematocrit values during the first week after inoculation. Serologic determinations on preinoculation and convalescent serum samples are still pending.

Of the monkeys that died after inoculation, those receiving 100 or 1,000 $MICLD_{50}$ developed pyrexia between 60 and 96 hr, and died 84-116 hr postinoculation (mean of 3 animals = 105 hr). Monkeys inoculated with 5 or 10 $MICLD_{50}$ of virus became pyrexic from 68-101 hr, and died from 108-164 hr postinoculation (mean of 8 animals = 138 hr).

The effect of decreasing the dose of Asibi virus administered was to prolong the course of the illness. The onset of pyrexia averaged 87 hr postinoculation in monkeys receiving 100 or 1,000 $MICLD_{50}$ and 82 hr in those given 5 or 10 $MICLD_{50}$. It is concluded that the use of lower doses of yellow fever virus than the standard 1,000 $MICLD_{50}$ dose will produce a fatal infection that has an extended experimental period, during which the effects of antiviral compounds could be assessed.

In an attempt to alter the course of VEE infection in mice, an experiment was run in which adult, male CD-1 strain mice were inoculated with 40,000 spleen cells (lymphocytes) from adult, female Fort Detrick strain white mice, and then inoculated by the same route with 10,000 median mouse intraperitoneal lethal doses ($MIPLD_{50}$) of Trinidad strain VEE from 30 min-96 hr after treatment. Mixtures of heterotypic lymphocytes are reported to release interferon for 48-72 hr when maintained *in vitro*. It was hoped that the administration of splenic heterotransplants would protect recipients against a lethal dose of VEE. There was no observed difference in mortality rates among treated and untreated mice receiving VEE virus in this experiment. Serum samples have been collected from treated, uninfected mice at various times after administration of heterotypic spleen transplants for interferon assay.

Work is progressing on the development of microtitration methods to run serum neutralization tests using a constant virus-varying serum dilution technique. This test is necessary if serial antibody responses of laboratory rodents are to be monitored. It is hoped that problems in obtaining the requisite cell cultures will be resolved in the immediate future.

Summary:

Two model systems were examined preparatory to testing antiviral agents. In the first, an inverse relationship between dosage of Asibi yellow fever virus and duration of clinical illness has been observed in rhesus monkeys. Thus, at the lower challenge doses, illness was prolonged sufficiently to permit testing of treatments. The incubation period did not change with virus dose.

In the second, no evidence of protection was observed in mice given splenic heterotransplants before infection with VEE virus.

Methods are being developed to perform plaque-reduction, serum-neutralization testing using microtitration equipment.

Publications:

None.

ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 02:	Prevention and Treatment of Biological Agent Casualties
Work Unit No. 096 02 412:	Develop Serological Methods for Military Vaccine Evaluation
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Division:	Virology
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Author:	Helen H. Ramsburg
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
	DA OL0913	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 05 21	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	02	412
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	CDOG 1212b(9)			

11. TITLE (Precede with Security Classification Code)*
(U) Develop Serological Methods for Military Vaccine Evaluation (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*
003500 Clinical Medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
71 05	CONT	DA	C. In-house

17. CONTRACT/GRANT
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER:*
c. TYPE: NA d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	0.5	10
	CURRENT 72	1	110

19. RESPONSIBLE DOD ORGANIZATION
NAME:* USA Medical Research Institute of Infectious Diseases
ADDRESS:* Fort Detrick, Md 21701

RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
NAME:* Virology Division USAMRIID
ADDRESS:* Fort Detrick, Md 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME:* Ramsburg, H. H.
TELEPHONE: 301 663-4111 Ext 7241
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME:
NAME: DA

21. GENERAL USE
Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Immunity; (U) Serology; (U) Tissue culture; (U) Plaque reduction; (U) Military medicine

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Develop a neutralization test in tissue culture which would be more sensitive and less costly to perform than the current mouse test.

24 (U) After selecting a cell-line, tests will be performed with various viruses to determine the optimal conditions for each.

25 (U) 71 05 - 71 06 - In determining the efficacy of an inactivated Eastern equine encephalomyelitis (EEE) vaccine, the hemagglutination-inhibition titers and serum neutralization (SN) indices were compared to the protection afforded animals challenged with virulent EEE virus. Only the SN indices were found to correlate with protection, therefore, the SN test in mice is being used to evaluate vaccines.

Since it has been reported in the literature that SN tests in tissue culture are found to be more sensitive, less costly, and quicker than the SN tests in mice, the development of a plaque reduction test has been initiated.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 096 02 412: Develop Serological Methods for Military Vaccine Evaluation

Description:

Develop a neutralization test in tissue culture which would be more sensitive and less costly to perform than the current mouse test.

Progress and Summary:

In determining the efficacy of an inactivated Eastern equine encephalomyelitis (EEE) vaccine, the hemagglutination-inhibition titers and serum neutralization (SN) indices were compared to the protection afforded animals challenged with virulent EEE virus. Only the SN indices were found to correlate with protection, therefore, the SN test in mice is being used to evaluate vaccines.

Since it has been reported in the literature that SN tests in tissue culture have been found to be more sensitive, less costly, and quicker than the SN tests in mice, the development of a plaque reduction test has been initiated.

Publications:

None.

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ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 02:	Prevention and Treatment of Biological Agent Casualties
Work Unit No. 096 02 800:	Development of a P valent *Staphylococcus aureus* Toxoid
Reporting Installation:	U. S. Army Medi ch Institute of Infectious Disea Fort Detrick, Ma
Division:	Pathology
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Authors:	Joseph F. Metzger, Colonel, MC Anna D. Johnson
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
				DA OL0895	71 07 01	[illegible]

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISB'N INSTR'N	8b. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
71 01 06	D. CHANGE	U	U	NA	NL	X YES ☐ NO	A. WORK UNIT

10. NO./CODES*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	02	800
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	CDOG 1212b(9)			

11. TITLE (Precede with Security Classification Code)* (U) Development of a Polyvalent Staphylococcus aureus Toxoid for Protection against an Illness of Military Importance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*
016800 Toxicology; 003500 Clinical Medicine; 004900 Defense

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
70 04	CONT	DA	C. In-house

17. CONTRACT GRANT

a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER*:
c. TYPE: NA d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	1	50
	CURRENT 72	1	39

19. RESPONSIBLE DOD ORGANIZATION

NAME:* USA Medical Research Institute of Infectious Diseases
ADDRESS:* Fort Detrick, Md 21701

RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION

NAME:* Pathology Division USAMRIID
ADDRESS:* Fort Detrick, Md 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME:* Metzger, J. F.
TELEPHONE: 301 663-4111 Ext 2134
SOCIAL SECURITY ACCOUNT NUMBER:

21. GENERAL USE
Foreign intelligence considered.

ASSOCIATE INVESTIGATORS
NAME:
NAME: DA

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Enterotoxin; (U) Staphylococcus; (U) Toxoid; (U) Military Medicine

23. TECHNICAL OBJECTIVE* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Develop a polyvalent toxoid which would include Staphylococcus aureus enterotoxins A, B, C, and D and other exoproteins.

24 (U) Preparation of a polyvalent toxoid of enterotoxins A, B, C, and D at a concentration of 500 micrograms per milliliter of each toxoid.

25 (U) 70 07 - 71 06 - A polyvalent toxoid was prepared utilizing purified enterotoxins B, B, and C. Residual toxicity was present in the safety test, but was not demonstrable by either immunological or biochemical procedures. Protection of monkeys varied from 33 - 100% after oral challenge with 40 - 400 micrograms per kilogram of purified enterotoxins.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

BODY OF REPORT

Project No. 1B662706A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662706A096 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 096 02 800: Development of a Polyvalent Staphylococcus aureus Toxoid

Description:

Develop a polyvalent toxoid which would include Staphylococcus aureus enterotoxins A, B, C, and D and other exoproteins.

Progress:

A 27-day, formalin-detoxified, crude, staphylococcal enterotoxin B (SEB) toxoid was prepared. To determine an optimum dosage and schedule for administration of the crude SEB toxoid, 2 schedules were tested for comparison to a known SEB toxoid.[1] Lot 87285 SEB toxoid, produced by Charles Pfizer Co., Terre Haute, Indiana, was used and simultaneously studied. All groups were challenged by the intravenous route with Lot 14-30 SEB toxin 6 weeks after the last dose of toxoid was administered. The groups, along with the dose, schedules, illness, and deaths are shown in Table I.

TABLE I. RESPONSE OF MONKEYS IMMUNIZED WITH EITHER CRUDE OR LOT 87285 AGAINST CHALLENGE WITH LOT 14-30 TOXIN

TOXOID	SCHEDULE	µg/kg	ILL/6	DEAD/6
Lot 87285	0.5 ml SC[a/]	10	[illegible]	0
	4 wk			
	0.5 ml SC	300	6	0
Lot 87285	0.1 ml ID[b/]	10	4	0
	3 wk			
	0.5 ml SC	300	6	1
Crude	0.5 ml SC	10	6	0
	4 wk			
	0.5 ml SC	300	6	3
Crude	0.1 ml ID	10	5	0
	3 wk			
	0.5 ml SC	300	6	4
Controls (No Toxoid)		10	6	0
		300	6	4

a. Subcutaneously.
b. Intradermally.

In order to study the effectiveness of the SEB toxoids against a more natural route of intoxication, immunized monkeys were challenged by gastric lavage (GL). This is accomplished by passing a 6-Fr pediatric catheter intranasally to the stomach. The toxin is then instilled in the stomach via the catheter. The catheter is flushed with 5 ml of sterile saline before removal. The monkeys were immunized with either Pfizer's Lot 87285 toxoid or with the crude toxoid. All monkeys were immunized using the same schedule: 0.1 ml intradermally (ID), followed in 3 weeks by 0.5 ml SC, and then 2 weeks later with 0.5 ml SC again. Six weeks after the last toxoid administration, the monkeys were challenged with 40 µg/kg [10 median illness doses (ID_{50})] of Lot 14-30 SEB toxin by GL. Because Lot 87285 toxoid (Pfizer) contains about 4 times as many µg of N_2 as does the crude toxoid, it was thought, for a better comparison, that one group of monkeys would be immunized using the Pfizer toxoid at a 1:4 dilution. The results are summarized in Table II.

TABLE II. EFFICACY IN MONKEYS OF LOT 87285 AND CRUDE SEB TOXOID AGAINST A GASTRIC CHALLENGE WITH LOT 14-30 TOXIN

TOXOID	CHALLENGE (40 μg/kg) Ill/Total
Crude	4/6
Lot 87285	1/5
Lot 87285 (1:4 dilution)	6/6
Controls	4/5

A trivalent material composed of purified enterotoxins A, B, and C was prepared at a final concentration of 500 μg/ml of each enterotoxin. Toxoiding with formalin was continued for 28 days. Excess formalin was removed by dialysis against merthiolated saline. The final product was filtered through a 0.2 μ millipore filter and bottled aseptically. Electrophoretically and immunologically no unaltered toxin was demonstrable. The product was safety tested using the technique previously described[27] with a modification. Instead of rectal thermocouple probes being used, a copper constantan thermister was implanted intramuscularly in the lumbar region for temperature monitoring. All monkeys given the trivalent toxoid had significant fever, emesis within 60-90 min after administration of the toxoid, and diarrhea. This trivalent toxoid was administered 0.1 ml ID on day 0, 0.5 ml SC on days 21 and 35 challenge was accomplished 6 weeks after the last injection.

TABLE III. RESPONSE OF MONKEYS IMMUNIZED WITH TRIVALENT ENTEROTOXOID

ENTEROTOXIN	ORAL CHALLENGE μg/kg	PROTECTION %
A	40	100
A	200	50
B	40	33
B	400	33
C	40	80
C	400	67

Though toxicity was demonstrated in the safety test, no adverse reactions were noted in immunized animals.

Summary:

A trivalent enterotoxoid was prepared utilizing purified enterotoxins A, B, and C. Residual toxicity remained as shown by safety tests although it was not demonstrable by other methods. Protection from oral challenge was greater against enterotoxins A and C than B.

Safety tests and immunizations were performed by Animal Assessment Division. Immunological studies were performed by Bacteriology Division.

Publications:

None.

LITERATURE CITED

1. U. S. Army Medical Research Institute of Infectious Diseases. 1 July 1970. Annual Progress Report, FY 1970, p. 139 to 144. Fort Detrick, Maryland.

2. U. S. Army Medical Research Institute of Infectious Diseases. 1 July 1969. Annual Progress Report, FY 1969, p. 87 to 102. Fort Detrick, Maryland.

ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 02:	Prevention and Treatment of Biological Agent Casualties
Work Unit No. 096 02 301:	Effects of Staphylococcal Enterotoxin B on Lymphoid Cells *in vitro*
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Division:	Pathology
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Author:	William H. Adler, Major, MC
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	DA OL0908	2. DATE OF SUMMARY	71 07 01	REPORT CONTROL SYMBOL				
3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISB'N INSTR'N	8b. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM	
70 12 22	D. CHANGE	U	U	NA	NL	[X] YES ☐ NO	A. WORK UNIT	

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	02	301
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1212b(9)			

11. TITLE (Precede with Security Classification Code) (U) Effects of Staphylococcal Enterotoxin B on Lymphoid Cells in vitro in BW Defense (U)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
010100 Microbiology; 003500 Clinical Medicine; 004900 Defense

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
70 12	CONT	DA	C. In-House

17. CONTRACT GRANT
a. DATES/EFFECTIVE: | EXPIRATION:
b. NUMBER: NA
c. TYPE: | d. AMOUNT:
e. KIND OF AWARD: | f. CUM. AMT.:

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	1	55
	CURRENT 72	1	66

19. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, Md 21701
RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
NAME: Pathology Division USAMRIID
ADDRESS: Fort Detrick, Md 21701
PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Adler, W. H.
TELEPHONE: 301 663-4111 Ext 2134
SOCIAL SECURITY ACCOUNT NUMBER
ASSOCIATE INVESTIGATORS
NAME:
NAME: DA

21. GENERAL USE
Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Toxin, Staphylococcal (SEB); (U) Lymphoid cells; (U) Pathogenesis; (U) Tissue culture; (U) Military Medicine

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Develop a microtechnique for the short-term culture of lymphoid cells and study in vitro the effects of SEB on these cells.

24 (U) Lymphoid cell culture allows the assay of the mitogenic properties of SEB, and the effects of the lymphoid mitogenic process on other tissue culture cell liver.

25 (U) 70 12 - 71 06 - A reproducible, uncomplicated, economical microtechnique for mouse or human lymphocyte culture has been established which uses a norbicarbonate buffer system which eliminates the need for an oxygen-air-carbon dioxide controlled humidity incubator and allows the use of any 37 C source.

A study of stimulation of lymphocytes with SEB has been initiated; it has been found that (a) SEB-stimulated lymphocytes are able to kill mouse fibroblasts and L-cells and (b) cell-free supernatants from SEB-stimulated lymphocyte cultures are toxic to other tissue culture cells.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 096 02 801: Effects of Staphylococcal Enterotoxin B on Lymphoid Cells *in vitro*

Description:

Develop a microtechnique for the short-term culture of lymphoid cells and study *in vitro* the effects of SEB on these cells.

Progress, Part I:

Previous methods for the culture of mouse and human lymphoid cells relied on a bicarbonate buffer system which required the use of a CO_2 incubator and controlled humidity[1,2,3/] (J. Exp. Med. 131:1049, 1970; J. Immun. 105:984 and 1453, 1970). Also, the number of cells cultured and the culture volume needed for a reproducible assay system was uneconomical in terms of the number of animals and the amount of blood needed, and the quantity of material used. A new technique was developed for the cell culture system. The buffer used in this system was Na-"HEPES" (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid) which was used as a 25 m molar solution in standard culture media. The use of this buffer did away with the necessity for the CO_2 incubator, and allowed the use of either a 37 C water bath or 37 C standard incubator with no gas or humidity controls since the cultures were maintained in sealed tubes. The microtechnique modification allowed 10-25 times more cultures to be initiated per mouse or per ml of human blood. Since culture volumes were reduced 90%, more cultures could be maintained per unit volume of media. The modification led to a savings in the amount of materials used.

Summary, Part I:

A dependable, reproducible, microtechnique was developed for the *in vitro* study of mouse and human lymphocytes, which did not need a bicarbonate buffer system and, therefore, the cultures could be maintained in any 37 C source.

Progress, Part II:

Using the new culture technique described above, the effects of staphylococcal enterotoxin B (SEB) on lymphoid cells were studied. It was found that SEB could stimulate lymphocytes in mouse spleen and thymus and in human peripheral blood. The SEB stimulated over 60% of the mouse spleen

mononuclear cells and 90% of human peripheral blood mononuclear cells as described previously.[3] The effect of SEB contact with these cells was to initiate both morphologic changes and mitotic cycles. These effects, and the number of cells able to be effected, were comparable qualitatively and quantitatively to the effects seen with other lymphocyte stimulants such as phytohemagglutinin.

The mitogenic effect of SEB on mouse spleen cells could be inhibited by prior immunization of the mice with SEB. During the period of time when the mouse would be theoretically synthesizing peak amounts of anti-SEB antibody, the _in vitro_ response of the spleen lymphocytes to SEB was markedly inhibited, and only large amounts of SEB _in vitro_ could stimulate the cells. It was also found that anti-SEB antibody added to the cultures could also inhibit SEB stimulation of the lymphocytes. Therefore, it could be hypothesized that the spleen cells from SEB-immunized mice could be secreting anti-SEB antibody, _in vitro_, which could block the stimulating action of SEB on the lymphocytes.

The findings that (1) SEB stimulates the majority of mouse spleen mononuclear cells and almost all human peripheral lymphocytes, (2) SEB stimulates mouse thymus cells, (3) the extent of stimulation is comparable to that seen with other nonspecific mitogens, and (4) that immunization of the donor animal with SEB does not increase the response of the donor's spleen cells to SEB _in vitro_, all show that SEB is acting as a nonspecific mitogenic stimulus rather than as an antigenic stimulus in its effects on lymphocytes.

Lymphocytes which have been stimulated by a variety of substances, both mitogens and antigens, release substances which can effect macrophages and lymphocytes, and can be toxic to tissue culture cell lines _in vitro_[4,5] (Mediators of Cellular Immunity, 1970; Fed. Proc. 30:647, 1971). Therefore, experiments were performed to determine if SEB-stimulated lymphocytes release toxic substances, or if the stimulated lymphocytes are toxic to cells of non-lymphoid origin.

It was found that lymphocytes, either human or mouse, stimulated by SEB, were able to inhibit mouse L cell and normal mouse fibroblast growth and were able to kill L cells and fibroblasts _in vitro_. These effects were measured either by studying the morphological changes in L cell or fibroblast cultures _in vitro_, determining cell death by trypan blue dye exclusion, or by measuring the release of ^{51}Cr from a previously tagged monolayer. Neither SEB alone, or unstimulated lymphocytes alone were able to affect L cell or fibroblast growth or viability, while the SEB stimulated lymphocytes killed a large percentage of the L cells or fibroblasts during a 48-hr incubation period.

The supernates from lymphocyte cultures, either unstimulated, or stimulated with SEB, were tested for their effect on the L cells and normal fibroblasts. The cell-free supernates from the SEB-stimulated lymphocyte cultures were able to inhibit L cell and fibroblast growth and killed a majority of either the L cells or fibroblasts as judged by the trypan blue staining characteristics and the growth characteristics of the cells in culture.

The supernate-toxic-factor is relatively heat insensitive, non-dialyzable, and appears at least in part to be associated with a protein fraction which has no enzyme activity. Work is progressing on further characterization of the factor.

Summary, Part II:

SEB stimulates lymphoid cells as a nonspecific mitogen. The stimulated lymphocytes are able to kill mouse L cells or syngenic normal mouse fibroblasts in vitro and the supernates of the SEB stimulated lymphocyte cultures are able to effect the growth and viability of mouse L cells or fibroblasts.

Whether these toxic effects of the stimulation of lymphocytes with SEB have any biologic significance is currently under investigation.

Publications:

None.

LITERATURE CITED

1. Adler, W. H., T. Takiguchi, B. Marsh, and R. T. Smith. 1970. Cellular recognition by mouse lymphocytes in vitro. I. Definition of a new technique and results of stimulation by phytohemagglutinin and specific antigens. J. Exp. Med. 131:1049-1078.

2. Adler, W. H., T. Takiguchi, B. Marsh, and R. T. Smith. 1970. Cellular recognition by mouse lymphocytes in vitro. II. Specific stimulation by histocompatibility antigens in mixed cell culture. J. Immun. 105:984-1000.

3. Peavy, D., W. H. Adler, and R. T. Smith. 1970. The mitogenic effects of endotoxin and staphylococcal enterotoxin B on mouse spleen cells and human peripheral lymphocytes. J. Immun. 105:1453-1458.

4. Landy, M., R. T. Smith, and H. S. Lawrence (ed.). 1970. Mediators of cellular immunity. Academic Press, New York.

5. Kaplan, J. 1971. Staphylococcal enterotoxin B induced release of macrophage migration inhibition factor from normal lymphocytes. Fed. Proc. 30:647 (Abstract).

ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 03:	Laboratory Identification of Biological Agents
Work Unit No. 096 03 006:	Early Immune Response In Infectious Diseases and Toxemia
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Division:	Bacteriology
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Author:	Joseph Kaplan, Major, MD
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA OL0870	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 01 06	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	03	006
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	CDOG 1212b(9); 1412a(2)			

11. TITLE (Precede with Security Classification Code)* **(U) Early Immune Response in Infectious Disease and Toxemia of Significance in BW Defense** (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS* 003500 Clinical Medicine; 016800 Toxicology; 004900 Defense

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
66 08	CONT	DA	C. In-house

17. CONTRACT GRANT
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER*:
c. TYPE: NA d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.:

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	1	60
	CURRENT 72	1	90

19. RESPONSIBLE DOD ORGANIZATION
NAME:* USA Medical Research Institute of Infectious Diseases
ADDRESS:* Fort Detrick, Md 21701
RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
NAME:* Bacteriology Division USAMRIID
ADDRESS:* Fort Detrick, Md 21701
PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME:* Kaplan, J.
TELEPHONE: 301 663-4111 Ext 7341
SOCIAL SECURITY ACCOUNT NUMBER:
ASSOCIATE INVESTIGATORS
NAME:
NAME: DA

21. GENERAL USE
Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Immunology; (U) Antibody formation; (U) Chemotaxis; (U) Hemagglutination; (U) Complement; (U) Macrophage; (U) Enterotoxin; (U) Staphylococcus; (U)Military Medicine

23. TECHNICAL OBJECTIVE* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Develop and employ in vitro methods of studying lymphocyte activation by antigens and toxins.

24 (U) Sensitivity of various assay techniques for detection of immunoglobulin, including chemotaxis, augmented hemagglutination, and macrophage migration will be determined. These will be applied to detection of circulating antibody and antibody forming cells early in the course of infection in animals and humans.

25 (U) 70 07 - 71 06 - Migration-inhibition factor (MIF) induced by staphylococcal enterotoxin B (SEB) was shown to be similar to antigen-induced MIF in that (a) its release was dependent on active cellular processes involving protein synthesis and (b) it was primarily localized in the albumin peak when chromatographed on Sephadex G-200. Delayed hypersensitivity in the guinea pig as measured by in vitro macrophage inhibitio was radiosensitive when tested 8 days after sensitization. At 13 days, it appeared to be relatively more radioresistant than humoral antibody production. Initial attempts to incorporate spin-labeled dihydrotestosterone into lymphocyte membranes were unsuccessful. Further studies are in progress to incorporate endogenously spin-labeled stearic acid into lymphocyte membranes.

Publications: Fed. Proc. 30:647, 1971
Infect. Immun. 3:94-99, 1971.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 03: Laboratory Identification of Biological Agents

Work Unit No. 096 03 006: Early Immune Response in Infectious Diseases and Toxemia

Description:

Develop and employ *in vitro* methods of studying lymphocyte activation by antigens and toxins.

Progress, Part I:

In the report last year[1/] (USAMRIID Annual Progress Report, FY 1970, p. 203), work was described which demonstrated that staphylococcal enterotoxin B (SEB) induced normal lymphocytes to release a migration-inhibition factor (MIF).

To determine whether SEB-induced release of MIF was an active cellular process, the effect of a metabolic inhibitor, mitomycin-C, was examined. As shown in Table I, mitomycin-C in a concentration of 10 μg/ml prevented release of MIF by lymphocytes cultured with SEB. This indicated that release of MIF by SEB-stimulated lymphocytes is an active cellular process.

TABLE I. EFFECT OF MITOMYCIN-C ON SEB-INDUCED RELEASE OF MIF FROM NORMAL LYMPHOCYTES

MIGRATION INDEX[a/] OF SUPERNATANT FLUID		
SEB	SEB + Mitomycin-C	Without added SEB (Control)
75*	102	100

a. Migration Index = $\frac{\text{Migration test supernatant}}{\text{Migration control supernatant}}$ X 100; assayed with purified macrophages from guinea pig peritoneal exudate cells.

* = $p < 0.05$

To characterize SEB-induced MIF, 20-fold concentrated supernatant fluids of SEB-stimulated and unsensitized lymphocyte cultures to which equivalent concentrations of SEB have been added terminally were examined by

Sephadex G-200 chromatography. Four peaks that corresponded to guinea pig macroglobulin, γ-G globulin, albumin and components with molecular weights <10,000 were observed with ultraviolet spectrophotometry.

Pooled, concentrated fractions representing each peak were assayed for SEB content by radioimmunodiffusion. A 1:5 dilution of each fraction was then tested for migration-inhibition activity against peritoneal exudate cells (Table II).

TABLE II. MACROPHAGE MIGRATION-INHIBITION BY SEPHADEX G-200 FRACTIONS OF SEB-STIMULATED LYMPHOCYTE SUPERNATANT FLUIDS

FRACTION	EXPERIMENT NO.	MIGRATION INDEX[a]
I	1	134, 102
	2	104, 91
	3	116
	1	126, 113
II	2	113, 102
	3	86
	1	89*, 74*
III	2[b]	
	3	75*
	1	119, 69*, 78*
IV	2	55*, 81*, 88
	3	84, 97

a. Each value represents a single assay

b. Test invalid due to lymphocyte contamination of macrophages

* = $p < 0.05$

The data represent migration indices for the 4 Sephadex fractions obtained in 3 separate experiments. Fraction III, the albumin fraction, was found to contain SEB (MW 28,000); therefore, this fraction was assayed with purified macrophages. Fractions I, II and IV did not contain SEB and were assayed with peritoneal exudate cells.

Fractions I and II did not demonstrate migration-inhibition. SEB-induced MIF activity, like antigen-induced MIF activity, was found in

Fraction III. In addition, 4 of 8 experiments with Fraction IV showed inhibition. This activity could represent: (a) an inhibitory factor unique for SEB-lymphocyte interaction; (b) an active fragment of MIF with higher molecular weight, or (c) a factor cytotoxic for macrophages.

These findings add to the growing evidence that lymphocytes can be "turned-on" by both specific antigenic stimulation and by many nonspecific stimulants, and once "turned on" will undergo stereotyped, precoded changes including the release of various effector molecules including macrophage MIF, lymphotoxin, lymphocyte transformation factor, macrophage chemotactic factor and antibody.

Summary, Part I:

SEB-induced MIF was shown to be similar to antigen induced MIF in that its release was dependent on active cellular processes involving protein synthesis and it is primarily localized in the albumin peak on a Sephadex G-200 chromatograph.

Progress, Part II:

In a separate series of experiments, the effect of irradiation on antibody production and *in vitro* macrophage migration was studied. The suppressive effect of X-irradiation on humoral antibody production has been well documented[2/] (J. Immun. 66:181, 1951). Previous studies have been shown that irradiated, immunized animals without detectable serum antibody retain the capacity to exhibit delayed type hypersensitivity as measured by skin testing[3/] (J. Exp. Med. 112:65, 1960). These studies suggested that delayed hypersensitivity was relatively radioresistant when compared to humoral antibody response. Macrophage migration-inhibition is the best correlate of *in vivo* delayed hypersensitivity. Consequently, this parameter was employed in the present studies to determine the effect of X-irradiation on delayed hypersensitivity.

Guinea pigs were given 300 rads X-irradiation and, 1 day later, were sensitized with bovine gamma globulin (BGG) in complete Freund's adjuvant. Control animals were sensitized but not irradiated. At varying times after sensitization, peritoneal exudate cells were obtained and allowed to migrate out of capillary tubes in culture media with or without BGG. At the same time, serum was obtained for measurement of anti-BGG titers as measured by a sensitive hemagglutination assay. Results are shown in Table III.

As expected, at both 8 and 13 days following BGG sensitization, humoral antibody responses of irradiated animals were markedly depressed. In keeping with findings reported in the literature,[3/] delayed hypersensitivity was demonstrable at day 13 in irradiated as well as control animals. Such evidence has been employed to support the thesis that induction of delayed

TABLE III. EFFECT OF X-IRRADIATION ON INDUCTION OF DELAYED HYPERSENSITIVITY TO BGG AS MEASURED BY MACROPHAGE MIGRATION-INHIBITION

TREATMENT	DAY[a]	NUMBER SHOWING MIGRATION-INHIBITION WITH BGG	HA TITER Median	HA TITER Range
X-Ray[b]	13	4/6	<1:10	<1:10-1:320
Control	13	6/6	1:80	1:20-1:1280
X-Ray	8	0/9*	<1:10	<1:10-1:10
Control	8	8/20	1:160	1:10-1:1280

a. Days after BGG sensitization

b. Animals irradiated 24 hr prior to BGG sensitization

* = $p < 0.05$

hypersensitivity is radioresistant. However, when irradiated animals were examined at 8 days, they showed no evidence of delayed hypersensitivity. This finding indicated that, like humoral antibody responses, the development of delayed hypersensitivity was sensitive to irradiation. The data also indicated that delayed hypersensitivity responses differed from humoral antibody responses in that the rate of recovery from irradiation damage is more rapid.

Irradiation suppression of macrophage-inhibition on day 8 may be due to either a failure of lymphocytes to produce MIF, or a failure of macrophages to respond to MIF, or both. Experiments are in progress to examine these possibilities.

<u>Summary, Part II</u>:

Delayed hypersensitivity in the guinea pig as measured by *in vitro* macrophage migration-inhibition was radiosensitive when tested 8 days after sensitization. At 13 days after sensitization, it appeared to be relatively more radioresistant than humoral antibody production.

Progress, Part III:

Experiments were initiated to determine whether membrane conformational changes are the initial triggering events which activate lymphocytes. It is planned to incorporate spin labels into living lymphocyte membranes, and examine spectral changes following specific and nonspecific lymphocyte stimulation by means of electron spin resonance (ESR) techniques.

Spin-labelled dihydrotestosterone was coupled with fatty acid-free bovine serum albumin (BSA) or sonicated in culture medium to increase its solubility. Complexing was demonstrated by a change in the characteristic ESR spectrum of a freely mobile molecule to that of a restricted molecule. Media containing steroid label-BSA complex or sonicated label was incubated for varying periods of time with guinea pig lymph node lymphocytes purified by discontinuous BSA-gradient ultracentrifugation. Following incubation, the cells were washed repeatedly.

The results of initial experiments suggested that cells had been successfully labelled. However, regardless of the number of cell washes, spin label was always found in cell-free wash fluid and had the same spectral characteristics as that seen in the packed cells. This suggested the possibility that label was loosely and reversibly bound to the cell membrane or that, following pinocytosis, it was readily extruded by the cell.

Studies are in progress to determine efficacy of using spin-labelled fatty acids in lymphocyte cultures in an attempt to endogenously incorporate spin label into cell membranes.

Summary, Part III:

Initial attempts to incorporate spin-labelled dihydrotestosterone into lymphocyte membranes were unsuccessful. Further studies are in progress to incorporate endogenously spin-labelled stearic acid into lymphocyte membranes.

Presentations:

1. Kaplan, J. SEB-induced release of macrophage migration inhibition factor from normal lymphocytes. Presented at Federation of American Societies for Experimental Biology, Chicago, 12-17 April 1971.

Publications:

1. Craig, C. P., S. J. Normann, V. McGann, and W. S. Irvin. 1971. Chemotactic activity generated by staphylococcal enterotoxin B. Infect. Immun. 3:94-99.

2. Kaplan, J. 1971. SEB-induced release of macrophage migration inhibition factor from normal lymphocytes. Fed. Proc. 30:647 (abstract).

LITERATURE CITED

1. U. S. Army Medical Research Institute of Infectious Diseases. 1 July 1970. Annual Progress Report, FY 1970. p. 203 to 209. Fort Detrick, Maryland.

2. Taliaferro, W. H., and L. G. Taliaferro. 1951. Effect of X-rays on immunity: A review. J. Immun. 66:181-212.

3. Uhr, J. W., and M. Scharff. 1960. Delayed hypersensitivity. V. The effect of x-irradiation on the development of delayed hypersensitivity and antibody formation. J. Exp. Med. 112:65-75.

ANNUAL PROGRESS REPORT

Project No. 3A061101A91C: In-house Laboratory Independent Research (U)
(1B662711A096): (Medical Defense Aspects of Biological Agents) (U)

Task No. 3A061101A91C 00:
(1B662711A096 03): (Laboratory Identification of Biological Agents)

Work Unit No. 91C 00 131: Mathematical and Computer Applications in
(096 03 008): Infectious Disease Research

Reporting Installation: U. S. Army Medical Research Institute of Infectious Diseases
Fort Detrick, Maryland

Division: Physical Sciences

Period Covered by Report: 1 July 1970 to 30 June 1971

Professional Authors: Robin T. Vollmer, Captain, MC
Darrell A. Leonhardt, SP4

Reports Control Symbol: RCS-MEDDH-288(R1)

Security Classification: UNCLASSIFIED

272

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
				DA OL0897	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 01 06	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	131
b. ~~CONTRIBUTING~~	62711A	1B662711A096	03/008	
c. ~~CONTRIBUTING~~	CDOG 1212b(9); 1412a(2)			

11. TITLE (Precede with Security Classification Code)*
(U) Mathematical and Computer Applications in Infectious Disease Research (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*
004900 Defense; 009700 Mathematics and Statistics; 003500 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
69 11	CONT	DA	C. In-house

17. CONTRACT GRANT
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER*:
c. TYPE: NA d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.:

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	1	16
	CURRENT 72	1	16

19. RESPONSIBLE DOD ORGANIZATION
NAME:* USA Medical Research Institute of Infectious Diseases
ADDRESS:* Fort Detrick, Md 21701
RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
NAME:* Physical Sciences Division USAMRIID
ADDRESS:* Fort Detrick, Md 21701
PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME:* Vollmer, R. T.
TELEPHONE: 301 663-4111 Ext 7291
SOCIAL SECURITY ACCOUNT NUMBER:
ASSOCIATE INVESTIGATORS
NAME:
NAME: DA

21. GENERAL USE
Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Diagnosis; (U) Computers; (U) Medicine; (U) Military Medicine

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Develop techniques for utilization of computers, statistics and mathematics to process and interpret scientific data.

24 (U) Automation of data analysis currently accomplished with the Fort Detrick CDC 3150 Computer is being expanded with a data acquisition system. The theories and disciplines of numerical analysis, differential equations, statistical tests of hypothesis, experimental design, information storage and retrieval, and pattern recognition are utilized to analyze and interpret data gathered by various investigators.

25 (U) 70 07 - 71 06 - Mathematical, statistical and computer techniques have been applied to data emanating from the Institute. The input has come from radioimmunoassays and studies of renal physiology, amino acids, volunteer studies, serum proteins, and cardiovascular parameters. The National Institutes of Health SAAM-25 program is being used in multicompartmental analysis of monkey lipid metabolic changes in infection.

Data from sandfly fever volunteer studies has been stored in a computer data bank. A computerized file has been created for monitoring the status of the special immunization program required for at-risk personnel.

A Rockefeller University program has been adapted for the Fort Detrick computer for determination of protein gradients produced during zonal centrifugation.

Plans are complete for procurement of a calculator-based data acquisition system.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3A061101A91C: In-house Laboratory Independent Research (U)
(1B662711A096): (Medical Defense Aspects of Biological Agents) (U)

Task No. 3A061101A91C 00:
(1B662711A096 03): (Laboratory Identification of Biological Agents)

Work Unit No. 91C 00 131: Mathematical and Computer Applications in
(096 03 008): Infectious Disease Research

Description:

Develop techniques for utilization of computers, statistics and mathematics to process and interpret scientific data.

Progress:

The computer programs devised for analysis of amino acid, renal physiologic data, and radioimmunoassay standard curves have been maintained and upgraded.

The National Institutes of Health SAAM-25 program is being used in the multicompartmental analysis of monkey lipid metabolic changes in infection.

The data from 3 volunteer sandfly fever projects have been stored in a computer data bank, and 2 more projects are being processed in a similar fashion. An analysis of covariance statistical model with mixed effects and partial replication is being explored for statistical hypothesis testing of this data.

A system of computer programs has been written up to calculate cardiovascular parameters and the statistical moments of these parameters.

A computerized immunization file has been created to contain all special immunizations and antibody titers recorded on personnel requiring protective immunization. This file will be used to list automatically each month those personnel requiring boosters and/or new assays for immunity. The file will, therefore, be available for research inquiries and preparation of summary reports on experimental vaccines.

Two stand-alone teletypes have been procured to automatically record data from 2 isotope counters. Computer programs have been developed to read the punched paper tape produced and perform the calculations necessary to analyze the data.

Plans have been completed for procurement of a calculator-based data acquisition system to record automatically, in a fashion similar to that for the isotope counting, the digitized analog data emanating from 2 amino acid analyzers and a densitometer. Again, punched paper tape will be the computer-compatible output of the system.

A computer program originating from the Rockefeller Institute has been adapted for use on the Fort Detrick computer. This program determines relative and absolute protein gradients produced during zonal centrifugation.

Summary:

Mathematical, statistical and computer techniques have been applied to data emanating from the Institute.

Publications

None

ANNUAL PROGRESS REPORT

Project No. 3A061101A91C: (1B662711A096):	In-house Laboratory Independent Research (U)
Task No. 3A061101A91C 00: (1B662711A096 03):	(Laboratory Identification of Biological Agents)
Work Unit No. 91C 00 132: (096 03 009):	Application of Electron Spin Resonance Spectroscopy to Infectious Disease Research
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Division:	Bacteriology
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Author:	William J. Caspary, Captain, MSC
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
	DA OL0898	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
70 07 21	D. CHANGE	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A061101A91C	00	132
b. CONTRIBUTING	62711A	1B62711A096	03/009	
c. CONTRIBUTING	CDOG 1212b(9); 1412a(2)			

11. TITLE (Precede with Security Classification Code)*
(U) Application of electron spin resonance spectrometry to infectious disease research (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*
004900 Defense; 003200 Biochemistry; 003500 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
69 11	CONT	DA	C. In-house

17. CONTRACT/GRANT
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER:*
c. TYPE: NA d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	1	44
	CURRENT 72	1	44

19. RESPONSIBLE DOD ORGANIZATION
NAME:* USA Medical Research Institute of Infectious Diseases
ADDRESS:* Fort Detrick, Md 21701

RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
NAME:* Physical Sciences Division USAMRIID
ADDRESS:* Fort Detrick, Md 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME:* Caspary, W. J.
TELEPHONE: 301 663-4111 Ext 7341
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Kanode, Jr., R. G.
NAME: DA

21. GENERAL USE
Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Military Medicine; (U) Spectroscopy; (U) Biochemistry; (U) Electron spin resonance; (U) Spin labels

23. TECHNICAL OBJECTIVE.* 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Establish usefulness of electron spin resonance spectroscopy in solving problems related to infectious diseases.

24 (U) Search for the effect of infectious agents and toxins on biochemical reactions involving free radicals. Spin label compounds involved in infectious or immunologic processes.

25 (U) 70 07 - 71 06 - Employing electron spin resonance spectroscopy techniques, we have established that: (1) nucleic acid can be spin labeled under mild conditions and the amount of label can be controlled. The degree of labeling desired for biological studies will be a compromise between spectrometer sensitivity and retention of biological activity; (2) the spectra of different nucleic acid biopolymers are different although not necessarily under all conditions; and (3) spectra from complex noninteracting mixtures can be resolved into their individual components.

Publications: Biophys. Soc. Abstracts 11:23a, 300a, 1971 (abstracts).

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3A061101A91C: In-house Laboratory Independent Research (U)
(1B662711A096):

Task No. 3A061101A91C 00:
(1B662711A096 03): (Laboratory Identification of Biological Agents)

Work Unit No. 91C 00 132: Application of Electron Spin Resonance
(096 03 009): Spectroscopy to Infectious Disease Research

Description:

Establish usefulness of electron spin resonance spectroscopy in solving problems related to infectious diseases.

Progress:

Although originally developed for the study of physical properties of electrons orbiting within an atom, it has appeared feasible to adopt the technique of electron spin resonance (ESR) spectroscopy to investigations of basic mechanisms of infection and immunity.

Paramagnetic molecules, or molecular carrying unpaired electrons, can be found as naturally occurring free radicals, relatively uncommon and usually unstable, or as metal complexes, e.g. in certain metallo-enzymes. In addition, paramagnetic molecules, termed spin labels, can be synthesized and incorporated as a small tag into larger molecules of biological interest. It was postulated that by utilizing spin labelled nucleic acids as electronic probes, it would be possible to obtain basic information regarding replication of viral nucleic acid in cells of infected hosts.

The spin label, 3-(2-iodoacetamido)-2,2,5,5-tetramethyl-1-pyrrolidinyl-oxyl, was reacted with various polynucleotides (Table I) and with denatured (d-DNA) and native desoxyribonucleic acid (DNA) under various mild conditions.

TABLE I. PROBABLE ATTACHMENT SITE OF THE SPIN LABEL REAGENT, 3-(2-IODOACETAMIDO)-2,2,5,5-TETRAMETHYL-L-PYRROLIDINYLOXYL, ON POLYNUCLEOTIDES.

POLYNUCLEOTIDE	PROBABLE SITE OF ATTACHMENT
$r(C)_n$	N (3)
$r(A)_n$	N (1)
$r(G)_n$	N (7)
$r(I)_n$	N (7)
$r(U)_n$	?

It was found that by varying the experimental conditions, the ratio of label to base could be changed. The conditions used were 25 C at pH 6.7 for 7 days in 40% ethanol-0.01 M phosphate (HMP) or 10 days in 60% ethanol-HMP, conditions A and B respectively. Results are shown in Table II.

TABLE II. EFFECT OF REACTION CONDITIONS ON YIELD OF SPIN LABELLED POLYNUCLEOTIDES.

	CHEMICAL YIELD (Spin Label: Base X 10^4)	
POLYNUCLEOTIDE (1×10^{-3} M)	Experimental Condition A[a]	Experimental Condition B[b]
rA	67	237
rU	3.6	14.9
rA·rU	2.6	
rI	7.7	8.8
rG	4.4	
rC	24.	152.
rG·rC	6.8	
Denatured DNA	25	100
Native DNA	2.8	4.2

a. Spin reagent Base = 5

b. Spin reagent Base = 10

In addition, we have determined that the spectrum of a small oligonucleotide (poly C) and RNAase resembled a free spin spectrum. Furthermore, we have been able to distinguish between the spectra of single stranded (poly C) and multistranded (poly I:C) polynucleotides. The effect of temperature on poly I:C could be followed using ESR techniques.

Generally speaking, the correlation time of the label (a measure of its immobility) was larger for double stranded nucleotides than for their single stranded analogs (Table III).

TABLE III. CORRELATION TIMES OF POLYNUCLEOTIDES AT 25 AND 37 C.

POLYNUCLEOTIDE[a]	CORRELATION TIME ($\times 10^9$ SEC) γ At 25 C	At 37 C
rA	2.2	1.3
rU	mixed	0.5
$rA^s \cdot rU$	3.7	2.2
$rA^s \cdot 2rU$	3.3	2.3
$rA \cdot rU^s$	2.7	1.7
$rA \cdot 2rU^s$	3.2	2.8 (?)
rI	1.6	1.1
rC	3.3	1.9
rG	≥10	≥10
$rI \cdot rC^s$	3.0	2.5
dDNA	2.8	1.2

a. Solvent was 0.15 M NaCl-HMP, pH 7.0.

Superscript "s" indicates the labelled portion of the molecule.

The effect of viscosity on denatured DNA was investigated to determine if weakly immobilized spectra were sensitive to conditions found in biological systems (Table IV). It may be seen that increased viscosity, from buffer to 20 or 30% sucrose, substantially increased correlation time.

In order to establish that the spectrum of a mixture of free spin and labelled polymers (noninteracting systems) can be resolved, the following study was performed. Correlation times for varying ratios of d-DNA to free spin label were determined and compared to similar values obtained from electronic simulation of the same conditions.

TABLE IV. EFFECT OF VISCOSITY ON CORRELATION TIME OF DENATURED DNA.

DILUENT[a]	VISCOSITY (centipoise)	CORRELATION TIME γ (X 10^9 sec)
Buffer	~0.80	1.2
Buffer - 20% sucrose	~1.50	1.8
Buffer - 30% sucrose	~2.40	3.6

a. Buffer was 0.15 M NaCl-HMP; temperature, 37 C.

Spectra of free spin and d-DNA were placed in separate memories of a Fabri-Tek signal averager. These spectra were mixed electronically in ratios of 1:3, 1:1 and 3:1. Simultaneously, samples were prepared in the same ratios (Table V).

TABLE V. COMPARISON OF EXPERIMENTALLY-DETERMINED AND COMPUTER-SIMULATED CORRELATION TIMES.

	CORRELATION TIME (γ)	
$\frac{\text{d-DNA}^s}{\text{FREE SPIN}}$ RATIO[a]	Experimentally Determined at 25 C	Electronically Simulated
∞[b]	3.3	(3.3)
3	0.9	0.7
1	0.35	0.3
.33	0.1	0.1
0[c]	0.05	(0.05)

a. Superscript "s" indicated labelled d-DNA

b. = pure d-DNA

c. 0 = free spin

Effective correlation times of spectra experimentally determined were in agreement with values obtained by electronic simulation, suggesting it is possible to resolve additive spectra.

Summary:

Employing electron spin resonance spectroscopy techniques, we have established that: (1) nucleic acid can be spin labelled under mild conditions and the amount of label can be controlled. The degree of labeling desired for biological studies will be a compromise between spectrometer sensitivity and retention of biological activity; (2) the spectra of different nucleic acid biopolymers are different although not necessarily under all conditions, and (3) spectra from complex noninteracting mixtures can be resolved into their individual components.

Presentations:

1. Caspary, W. J. Radical formation of carcinogenic hydrocarbons and their reaction with purine nucleosides. Presented at Biophysical Society Annual Meeting, New Orleans, La. 16-18 February 1971.

2. Caspary, W. J. Spin-labelled polynucleotides. Presented at Biophysical Society Annual Meeting, New Orleans, La. 16-18 February 1971.

Publications:

1. Caspary, W. J., B. I. Cohen, S. A. Lesko, and P. O. P. Ts'o. 1971. Radical formation of carcinogenic hydrocarbons and their reaction with purine nucleosides. Biophys. Soc. Abstracts 11:300a (abstract).

2. Caspary, W. J., L. M. Stempel, and P. Ts'o. 1971. Spin-labelled polynucleotides. Biophys. Soc. Abstracts 11:23a (abstract).

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ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 03:	Laboratory Identification of Biological Agents
Work Unit No. 096 03 010:	Trace Metal Changes During Infectious Diseases
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Division:	Physical Sciences
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Authors:	Robert S. Pekarek, Ph.D., (I, II, III, IV) William R. Beisel, M.D., (I, II, IV) Robert W. Wannemacher, Jr., Ph.D., (I, II, III) Peter J. Bartelloni, Lt. Colonel, MC, (I, IV) Jean B. DuBuy, Major, MC, (II) Glenn E. Marrs, Jr., Captain, VC (IV)
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA OL0899	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 01 06	D. CHANGE	U	U	NA	NL	[x] YES [ ] NO	A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	03	010
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	CDOG 1212b(9); 1412a(2)			

11. TITLE (Precede with Security Classification Code)*
(U) Trace Metal Metabolism during Infectious Disease of Military Medical Importance (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*
002300 Biochemistry; 003500 Clinical Medicine; 004900 Defense

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
67 01	CONT	DA	C. In-house

17. CONTRACT GRANT
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER:*
c. TYPE: NA d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	2	70
	CURRENT 72	2	88

19. RESPONSIBLE DOD ORGANIZATION
NAME:* USA Medical Research Institute of Infectious Diseases
ADDRESS:* Fort Detrick, Md 21701
RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
NAME:* Physical Sciences Division USAMRIID
ADDRESS:* Fort Detrick, Md 21701
PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME:* Pekarek, R. S.
TELEPHONE: 301 663-4111 Ext 5214
SOCIAL SECURITY ACCOUNT NUMBER:
ASSOCIATE INVESTIGATORS
NAME: DuBuy, J. B.
NAME: Marrs, Jr., G. E. DA

21. GENERAL USE
Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Trace metals; (U) Endogenous mediator; (U) Bacterial infections; (U) Viral infection; (U) Toxemia; (U) Animal models; (U) Military medicine

23. TECHNICAL OBJECTIVE.* 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Assess trace metal changes during infectious illness as a possible aid in early identification of disease etiology.

24 (U) Measure serum trace metals during infectious illness of laboratory animals and man and determine mechanisms responsible for observed changes.

25 (U) 70 07 - 71 06 - Early significant decreases in serum Fe and Zn levels with a concomitant rise in serum Cu were demonstrated in humans infected with either acute bacterial or viral infections.

Recent animal studies have shown that infection-induced alterations in trace metal metabolism are initiated by an endogenous mediator released by neutrophils. Isotopic studies with Zn-65 have shown that the endogenous mediator protentiates the redistribution of Zn within the host.

Normal baseline values and ranges for serum Fe, Zn and Cu are being established for the rhesus monkey.

Publications: J. Lab. Clin. Med. 76:293-303, 1970.
Proc. Soc. Exp. Biol. Med. 136:584-587, 1971.
Fed. Proc. 30:643, 1971 (abstract).
Internat. Rev. Neurobiol. 14: 1971, In press.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 03: Laboratory Identification of Biological Agents

Work Unit No. 096 03 010: Trace Metal Changes During Infectious Disease

Description:

Assess trace metal changes during infectious illness as a possible aid in early identification of disease etiology.

Progress, Part I:

Recent prospective clinical studies in volunteers by this laboratory have demonstrated that significant alterations in the metabolism of Fe, Zn, and Cu occur early after exposure to either an acute bacterial (*Francisella tularensis* - Medical Division Project FY 68-4) or viral (attenuated Venezuelan equine encephalomyelitis vaccine - Medical Division Project FY 69-1 and sandfly fever virus, Sicilian type, Medical Division Project FY 70-1) infections[1-4] (Amer. J. Med. Sci. 258:14, 1969; USAMRIID Annual Progress Rep. FY 1970, p. 221; J. Lab. Clin. Med. 76: 293, 1970; Metabolism 1971, In press). Such alterations include marked depressions in both serum Fe and Zn concentrations with a concomitant rise in serum Cu.

In a more recent cooperative investigation with Drs. Hornick and DuPont at the University of Maryland School of Medicine (Contract No. DA-49-193-MD-2867), sequential changes in serum Zn and Cu concentrations were studied in volunteers following experimentally induced typhoid fever. Significant depressions in serum Zn concentrations were observed by the 3rd day, with maximal depression occurring around day 13, during the time of maximal febrile illness. By contrast serum Cu concentrations began to rise significantly on day 9 with the onset of febrile illness, reaching maximal levels (161% of control values) on day 14. Unlike serum Zn concentrations, which returned to normal with the remission of fever (day 30), serum Cu concentrations still remained significantly elevated.

Similar early depressions in serum Zn concentrations were observed in a group of volunteers studied prospectively after oral exposure to living adenovirus type 21 vaccine (Medical Division Project FY 71-2). Serum Zn concentrations were depressed as early as day 2 and remained significantly depressed throughout the study (to day 28). However, unlike the previous studies, no significant change in serum Cu concentrations was observed.

Currently, serum Fe, Zn, and Cu concentrations are being serially determined for evaluation in a group of volunteers who have been exposed to the 17-D strain of yellow fever vaccine (Medical Division Project FY 71-3). The prospective nature of the above studies and the rapid development of altered serum metal metabolism, even in exposed subjects who remained asymptomatic, suggest that these alterations in the prodromal period represent an early host response to the presence of invading microorganisms. Thus infection-induced changes and patterns of change in trace metal metabolism may have significance in providing new approaches to diagnosis and to an improved understanding of the various metabolic responses during infectious illness.

Summary, Part I:

Significant alterations in Fe, Zn, and Cu continue to be demonstrated in man after exposure to a variety of acute viral and bacterial infections. These alterations were shown to be an early host response, with the magnitude of these changes showing a close relationship to the duration and severity of febrile illness.

Progress, Part II:

Depressions in serum Fe and Zn concentrations, similar to those observed in man, were observed in a variety of laboratory animals following experimentally-induced infections or the administration of endotoxin or synthetic double-stranded RNA compounds[1/,5-7/] (Appl. Microbiol. 18:482, 1969; CES Annual Report, FY 1970, p. 13; Proc. Soc. Exp. Biol. Med. 136:584, 1971). Further studies from our laboratory (Part III), as well as those reported in the literature, indicated that the very early decreases in serum Zn and Fe were not the result of either increased excretion or decreased gastrointestinal absorption, but were due to a rapid redistribution of the two metals to cells of the reticuloendothelial system (RES). Further studies in animal models have shown that the infection and stress-induced alterations in Fe and Zn metabolism were mediated by an endogenous humoral factor released in part by neutrophiles[8-10/] (Fed. Proc. 28:691, 1969; Amer. J. Physiol. 216:1287, 1969; Proc. Soc. Exp. Biol. Med. 134:1150, 1970). This endogenous factor of serum Zn and Fe depression was shown to be present in the serum of a variety of laboratory animals within 2 hr after infection or intoxication, and in sufficient concentration so that its Zn depressing effect could be transferred to normal or endotoxin-tolerant recipient animals of the same or even different species. Endogenous mediator obtained from peritoneal leukocytes of various laboratory animals were shown to produce significant dose-related depressions in serum Zn and Fe concentrations in normal, endotoxin tolerant, or hypophysectomized animals.[1-8/]

Preliminary data from our laboratory characterizes the partially purified mediator isolated from both 2-hr serum and from peritoneal leukocytes to be heat labile, non-dialyzable, low molecular weight proteins (20-30,000 MW), which are soluble in organic solvents[11,12/] (Fed. Proc. 29:297, 1970; Bact. Proc. 1970, p.81). These initial data suggest that the

endogenous mediator present within 2 hr in the serum may be released, in part, by polymorphonuclear leukocytes. Although similar in some respects to endogenous pyrogen, the endogenous mediator of serum Zn and Fe depression does not show the species specificity that has been shown for endogenous pyrogen.

Since the endogenous mediator has been shown to lack species specificity, we attempted to demonstrate the presence of this mediator in the serum during a bacterial infection in man. While doing a joint study with Dr. Schwartz and associates at the University of Maryland, control serum samples and samples obtained on the first day of febrile illness in a group of volunteers infected with Salmonella typhosa were filtered through a Millipore filter and injected into rats intraperitoneally (IP). The postinfection serum produced significant depressions in both serum Fe and Zn concentrations in recipient animals when compared to NaCl inoculated controls or rats receiving control serum. When the postinfected serum was heated at 90 C for 30 min, the Zn depressing effect was lost. Thus the endogenous Zn depressing factor appears to be present in the serum of a febrile individual, and the fact that this effect can be transferred to a laboratory animal may prove to have potential diagnostic value.

Current studies on the further characterization of the infection- and stress-induced endogenous mediator of altered Fe and Zn metabolism have shown that the in vitro addition of these trace metals and other divalent cations inhibit or partially inhibit its in vivo serum Zn and Fe depressing effects. Unlike Fe, the depressing effect of the mediator could be blocked completely only by the addition of Zn. Although such binding studies suggest that the mediator may act as a chelating agent by facilitating the removal of these metals from the serum, a given amount of this mediator cannot account for the amount of metal lost from the serum when administered in vivo. Consequently, to state that the endogenous mediator acts solely as a chelating agent would be premature; however, in this regard, a possibility does exist. The parenteral or IP administration of the mediator may induce the release of more endogenous mediator as has been shown for endogenous pyrogen. Another possible mode of action is that the mediator also may act as a hormone-like substance eliciting its effect directly on the RES, either by promoting the increased uptake of Zn and Fe in these tissues or by inhibiting their release. Current studies are now in progress to further characterize the mechanism of action, as well as the ability to mediate changes in other biochemical and metabolic parameters.

Summary, Part II:

It has been demonstrated that the depressions of serum Zn and Fe during infection and other stresses in various laboratory animals are mediated by an endogenous factor released, in part, by neutrophiles. The mediator was shown to be a heat labile, nondialyzable, low molecular weight protein, and, it has been demonstrated to be present in human serum during febrile illness. In

defining the mechanism by which the mediator may act, it was shown that partially purified preparations of the mediator could bind both Zn and Fe. Further the in vivo addition of these divalent cations to the mediator could block or partially block its serum Fe and Zn depressing effects in vivo.

Progress, Part III:

Recent isotopic studies employing ^{65}Zn have been conducted in order to compare the distribution of this metal in the serum and various organs of the rat before and after the administration of the endogenous mediator. After a 24-hr pulse lable with 5 μCi/100 gm body weight given IP, the isotope was rapidly deposited in the liver, followed in descending order of concentration in the spleen, kidney, pancreas, lung, heart and blood. When the endogenous mediator was administered as 100 μg protein/ml IP 6 hr prior to sacrifice (18 hr after the ^{65}Zn pulse) significant increases in the uptake of Zn were observed in the liver, pancreas and remaining viscera of the gastrointestinal with a significant decrease in the serum and carcass (less internal organs and viscera). Thus a definite redistribution of Zn, independent of increased excretion or decreased gastrointestinal absorption, was induced by the mediator.

Summary, Part III:

Isotopic studies employing ^{65}Zn demonstrate that the endogenous mediator produces a redistribution of Zn in the tissues when administered to the host.

Progress and Summary, Part IV:

Serum zinc concentrations were determined repeatedly in 99 healthy young adult males by a simple automated atomic absorption spectrophotometric method. The mean serum Zn concentration of these normal subjects was 102 μg/100 ml (SD ± 17) with a calculated range of 68 to 136 μg/100 ml. When determined on a day-to-day basis, 95% of the values from a given individual were within 18 μg/100 ml of his own mean value.

Similar studies are now being conducted in order to establish normal baseline values and ranges for serum Fe, Zn, and Cu in the rhesus monkey.

Presentations:

1. Beisel, W. R. Changes in zinc metabolism during infection. Presented at Workshop on Zinc in Human Nutrition, The Food and Nutrition Board. National Research Council, Washington, D. C. 4-5 December 1970.

2. Beisel, W. R. Influence of acute infection on the metabolism of zinc and other trace elements. Presented at the American College of Neuropsychopharmacology, San Juan, P.R., 9-11 December 1970.

3. Pekarek, R. S. Further characterization of the endogenous mediator(s) of serum zinc and iron depression during infection and other stresses. Presented at Federation of American Societies for Experimental Biology, Chicago, Ill. 13-17 April 1971.

4. Beisel, W. R. Interrelated changes in host metabolism during infectious diseases. Presented at Workshop on Malabsorption, National Research Council, Washington, D. C. April 1971.

Publications:

1. Pekarek, R. S., G. A. Burghen, P. J. Bartelloni, F. M. Calia, K. A. Bostian, and W. R. Beisel. 1970. The effect of live attenuated Venezuelan equine encephalomyelitis virus vaccine on serum iron, zinc, and copper concentrations in man. J. Lab. Clin. Med. 76:293-303.

2. Pekarek, R. S. 1971. Effect of synthetic double-stranded RNA on serum metals in the rat, rabbit, and monkey. Proc. Soc. Exp. Biol. Med. 136:584-587.

3. Pekarek, R. S., and W. R. Beisel. 1971. Further characterization of the endogenous mediator(s) of serum zinc and iron depression during infection and other stresses. Fed. Proc. 30:643 (abstract).

4. Beisel, W. R., and R. S. Pekarek. 1971. Acute stress and trace element metabolism. International Rev. of Neurobiology, 14: (in press).

5. Wannemacher, R. W., Jr., R. S. Pekarek, P. J. Bartelloni, R. T. Vollmer, and W. R. Beisel. 1970. Changes in individual plasma amino acids following experimentally-induced sandfly fever virus infection. Metabolism (in press).

LITERATURE CITED

1. Pekarek, R. S., K. A. Bostian, P. J. Bartelloni, F. M. Calia, and W. R. Beisel. 1969. The effects of Francisella tularensis infection on iron metabolism in man. Amer. J. Med. Sci. 258:14-25.

2. U. S. Army Medical Research Institute of Infectious Diseases. 1 July 1970, Annual Progress Report, FY 1970. p. 221 to 230. Fort Detrick, Maryland.

3. Pekarek, R. S., G. A. Burghen, P. J. Bartelloni, F. M. Calia, K. A. Bostian, and W. R. Beisel. 1970. The effect of live attenuated Venezuelan equine encephalomyelitis virus vaccine on serum iron, zinc, and copper concentrations in man. J. Lab. Clin. Med. 76:293-303.

4. Wannemacher, R. W., Jr., R. S. Pekarek, P. J. Bartelloni, R. T. Vollmer, and W. R. Beisel. 1970. Changes in individual plasma amino acids following experimentally-induced sandfly fever virus infection. Metabolism (in press).

5. Pekarek, R. S., and W. R. Beisel. 1969. Effect of endotoxin on serum zinc concentrations in the rat. Appl. Microbiol. 18:482-484.

6. Commission on Epidemiological Survey. Dec. 1970. p. 13 to 21. In Annual Report, FY 1970, to the Armed Forces Epidemiological Board, Fort Detrick, Maryland.

7. Pekarek, R. S. 1971. Effect of synthetic double-stranded RNA on serum metals in the rat, rabbit, and monkey. Proc. Soc. Exp. Biol. Med. 136:584-587.

8. Pekarek, R. S., and W. R. Beisel. 1969. Zinc depressing effects of endotoxin and leukocytic pyrogen in the rat. Fed. Proc. 28:691 (abstract).

9. Kampschmidt, R. F., and H. Upchurch. 1969. Lowering of plasma iron concentration in the rat with leukocytic extracts. Amer. J. Physiol. 216:1287-1291.

10. Kampschmidt, R. F., and H. F. Upchurch. 1970. The effect of endogenous pyrogen on the plasma zinc concentration of the rat. Proc. Soc. Exp. Biol. Med. 134:1150-1152.

11. Pekarek, R. S., and W. R. Beisel. 1970. Characterization of the infection-induced endogenous mediator of serum zinc depression. Fed. Proc. 29:297 (abstract).

12. Pekarek, R. S., and W. R. Beisel. 1970. Endogenous mediator of serum iron depression during infection and endotoxemia. Bact. Proc. p. 81.

ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 03:	Laboratory Identification of Biological Agents
Work Unit No. 096 03 011:	Chemical Mediators of Infection
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Disease Fort Detrick, Maryland
Division:	Physical Sciences
Period Covered by Report:	1 February 1971 to 30 June 1971
Professional Author:	Jean B. duBuy, Major, MC
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
	DA OL0910	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 03 16	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	03	011
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1212b(9)	1412a(2)		

11. TITLE (Precede with Security Classification Code)*
(U) Chemical Mediators of Infection of Military Medical Importance (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*
003500 Clinical Medicine; 002300 Biochemistry; 004900 Defense

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
71 02	CONT	DA	C. In-house

17. CONTRACT/GRANT
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER:*
c. TYPE: NA d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	2	60
	CURRENT 72	1	84

19. RESPONSIBLE DOD ORGANIZATION
NAME:* USA Medical Research Institute of Infectious Diseases
ADDRESS:* Fort Detrick, Md 21701
RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
NAME:* Physical Sciences Division USAMRIID
ADDRESS:* Fort Detrick, Md 21701
PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME:* DuBuy, J. B.
TELEPHONE: 301 663-4111 Ext 6130
SOCIAL SECURITY ACCOUNT NUMBER:
ASSOCIATE INVESTIGATORS
NAME: Beisel, W. R.
NAME:
DA

21. GENERAL USE
Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Rabbits; (U) Purification; (U) Acrylamide gel electrophoresis; (U) Endogenous pyrogen; (U) Military Medicine

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Purify and characterize several chemical mediators of infection.

24 (U) Polyacrylamide gel electrophoresis is applied to various specimens; resulting fractions are characterized by various applicable methods.

25 (U) 71 03 - 71 06 - Purification of one of the chemical mediators of infection, endogenous pyrogen, has proceeded through the use of a modification of acrylamide gel electrophoresis allowing the separation of large amounts of protein on a preparative basis. Rabbit peritoneal exudates have been prepared as a source of endogenous pyrogen. Using an exudate of unusually high activity, it has been possible to obtain pyrogenic activity from one or two sections of the gel while other sections are inactive. Current efforts are directed toward maximizing the yield of pyrogen in exudates in order to obtain sufficient amounts of pyrogen to further assay purity and begin physicochemical characterization.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 01: Laboratory Identification of Biological Agents

Work Unit No. 096 03 011: Chemical Mediators of Infection

Description:

Purify and characterize several chemical mediators of infection.

Progress:

Purification of one of the chemical mediators of infection, endogenous pyrogen, has proceeded through the use of a modification of acrylamide gel electrophoresis allowing the separation of large amounts of protein on a preparative basis. Rabbit peritoneal exudates have been prepared as a source of endogenous pyrogen. Using an exudate of unusually high activity, it has been possible to obtain pyrogenic activity from one or two sections of the gel while other sections are inactive. Current efforts are directed toward maximizing the yield of pyrogen in exudates in order to obtain sufficient amounts of pyrogen to assay further its purity and begin physicochemical characterization.

Summary:

Pyrogenic activity can be localized to a discrete area following acrylamide gel electrophoresis of granulocytic pyrogen from rabbit peritoneal exudate supernatant fluids.

Publications:

None

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ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 01:	Laboratory Identification of Biological Agents
Work Unit No. 096 03 012:	Evaluation of Serum Glycoprotein Changes in Early Diagnosis of Infectious Illness
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Division:	Physical Sciences
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Author:	Gary L. Cockerell, Captain, VC
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
	DA OL0861	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 04 20	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	03	012
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	CDOG 1212b(9); 1412a(2)			

11. TITLE (Precede with Security Classification Code)* (U) Evaluation of Serum Glycoprotein Changes in Early Diagnosis of Infectious Illness of Medical Importance (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*
003500 Clinical Medicine; 004900 Defense; 003200 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
61 07	CONT	DA	C. In-house

17. CONTRACT GRANT
a. DATES/EFFECTIVE | EXPIRATION
b. NUMBER*
c. TYPE NA | d. AMOUNT
e. KIND OF AWARD | f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	1	50
	CURRENT 72	1	70

19. RESPONSIBLE DOD ORGANIZATION
NAME:* USA Medical Research Institute of Infectious Diseases
ADDRESS:* Fort Detrick, Md 21701
RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
NAME:* Physical Sciences Division USAMRIID
ADDRESS:* Fort Detrick, Md 21701
PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME:* Cockerell, G. L.
TELEPHONE: 301 663-4111 Ext 2104
SOCIAL SECURITY ACCOUNT NUMBER:
ASSOCIATE INVESTIGATORS
NAME:
NAME: DA

21. GENERAL USE
Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Glycoproteins; (U) Electrophoresis; (U) Infectious diseases; (U) Protein-bound carbohydrate; (U) Military medicine

23. TECHNICAL OBJECTIVE.* 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Evaluate biological activity, role and significance of serum glycoproteins in the early diagnosis of infectious disease.

24 (U) Determine glycoprotein electrophoretic patterns in normal and infected states using improved techniques, in order to understand their significance.

25 (U) 70 07 - 71 06 - Methods are being developed to allow a better quantitative and qualitative description of serum glycoproteins. When 1 ml of turpentine is inoculated subcutaneously into a rat, there is a nearly 2-fold increase 48 hr later in the percentage of alpha-2 and beta glycoglobulins. This system promises to be a convenient model to mimic changes seen in circulating glycoglobulins in infectious disease.

Publication: J. Infect. Dis. 122:329-334, 1970.

*Available to contractors upon originator's approval

DD FORM 1498, 1 MAR 68 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 01: Laboratory Identification of Biological Agents

Work Unit No. 096 03 012: Evaluation of Serum Glycoprotein Changes in Early Diagnosis of Infectious Illness

Description:

Evaluate biological activity, role and significance of serum glycoproteins in the early diagnosis of infectious illness.

Progress:

Current methods in use at USAMRIID for characterization of serum glycoproteins are limited in that one can only obtain the percentage distribution in the major serum fractions, i.e., albumin, α_1, α_2, β, and γ[1/] (Amer. J. Clin. Path. 50:137, 1968). Current efforts in this laboratory, therefore, have been directed toward development of assays for the 4 major protein-bound carbohydrate moieties, hexoses, hexosamines, sialic acids and methylpentoses, as a means of quantitating total protein-bound carbohydrate which can then serve as an indirect measure of total serum glycoprotein.

Preliminary results indicate that the new densitometer is recording linear concentrations of serum protein and glycoprotein from electrophesed cellulose acetate strips. It is hoped that this will give us a more accurate and rapid method of quantitation and eventually calculation and storage of information in computerized form.

Initial experiments confirm published information[2/] (Cancer Res. 27:2055, 1967) regarding the sterile subcutaneous turpentine abcess in the rat as a means of producing changes in circulating glycoglobulins. We are able to produce a nearly 2-fold relative increase in the percentage of α_2, and β glycoglobulins 48 hr after injection of 1 ml turpentine subcutaneously in the rat. This may prove to be one of the most convenient experimental models to mimic changes in serum glycoprotein levels seen in infectious illness.

Summary:

Methods are being developed to improve characterization of serum glycoproteins. A sterile, subcutaneous turpentine abcess in the rat appears to be a good model to mimic changes seen in circulating glycoglobulins in infectious disease.

Publications:

Rollins, J. B., G. A. Burghen, and W. R. Beisel. 1970. The influence of altered viral virulence on the response of host serum $alpha_2$ glycoglobulins. J. Infect. Dis. 122:329-334.

LITERATURE CITED

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2. Sarcione, E. J. 1967. Hepatic synthesis and secretory release of plasma alpha-2 (acute phase)-globulin appearing in malignancy. Cancer Res. 27:2025-2033.

ANNUAL PROGRESS REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 03: Laboratory Identification of Biological Agents

Work Unit No. 096 03 013: Serum Protein and Enzyme Changes for Diagnosis in Infection

Reporting Installation: U. S. Army Medical Research Institute of Infectious Diseases
Fort Detrick, Maryland

Division: Physical Sciences

Period Covered by Report: 1 July 1970 to 30 June 1971

Professional Authors: Michael C. Powanda, Captain, MSC
Robert W. Wannemacher, Jr., Ph.D.
Gary L. Cockerell, Captain, VC

Reports Control Symbol: RCS-MEDDH-288(R1)

Security Classification: UNCLASSIFIED

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
	DA OL0912	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 04 23	D. CHANGE	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	03	013
b. CONTRIBUTING				
c. ~~//////~~	CDOG 1212b(9); 1412a(2)			

11. TITLE (Precede with Security Classification Code)* (U) Serum Protein and Enzyme Changes for Diagnosis of Militarily Important Infections (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*
003500 Clinical Medicine; 004900 Defense; 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
71 04	CONT	DA	C. In-house

17. CONTRACT/GRANT
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER*:
c. TYPE: NA d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.:

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	2	8
	CURRENT 72	2	33

19. RESPONSIBLE DOD ORGANIZATION
NAME:* USA Medical Research Institute of Infectious Diseases
ADDRESS:* Fort Detrick, Md 21701
RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
NAME:* Physical Sciences Division USAMRIID
ADDRESS:* Fort Detrick, Md 21701
PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME:* Powanda, M. C.
TELEPHONE: 301 663-4111 Ext 6130
SOCIAL SECURITY ACCOUNT NUMBER:
ASSOCIATE INVESTIGATORS
NAME: Wannemacher, R. W.
NAME: Cockerell, G. L. DA

21. GENERAL USE
Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Proteins; (U) Enzymes; (U) Infectious disease; (U) Electrophoresis; (U) Military Medicine

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Evaluate changes in serum proteins and isoenzymes occurring during infectious disease and elucidate the kinetics of such changes.

24 (U) Using polyacrylamide gel electrophoresis, measure changes in serum proteins and enzymes during infections.

25 (U) 71 04 - 71 06 - An evaluation of the suitability of polyacrylamide gel electrophoresis for analysis of serum proteins and enzymes was begun recently. Current efforts are directed toward determining the optimal conditions for sample analysis.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 03: Laboratory Identification of Biological Agents

Work Unit No. 096 03 013: Serum Protein and Enzyme Changes for Diagnosis in Infection

Description:

Evaluate changes in serum proteins and isoenzymes occurring during infectious disease and elucidate the kinetics of such changes, as an aid to early diagnosis.

Progress:

Changes in circulating levels of certain classes of serum proteins have been noted in both experimentally induced and naturally acquired infectious illness of varying etiology[1-6] (Proc. Soc. Exp. Biol. Med. 101:340, 1959; Amer. J. Clin. Path. 42:1, 1964; J. Lab. Clin. Med. 72:794, 1968; Arch. Intern. Med. 123:620, 1969; New Engl. J. Med. 281:1081, 1969; J. Infect. Dis. 122:329, 1970). Some form of paper or cellulose acetate electrophoresis had been used in all of these studies. The resolving power of these systems limits one's ability mainly to that of detecting changes in classes of serum proteins rather than in individual substances. At least 29 proteins of human serum have been physiochemically defined[7] (Molecular Biology of Human Proteins, p. 173, 1966). This number does not include the γ globulins, the multitude of circulating enzymes, the clotting factors or the components of complement. One method whereby the specific proteins which give rise to the changes in serum protein classes in infectious diseases can be identified is polyacrylamide gel electrophoresis[8] (Ann. N.Y. Acad. Sci. 121:404, 1964).

Serum enzyme tests have aided in the diagnosis of myocardial infarction, pancreatitis, acute hepatitis, toxic liver damage and muscular dystrophy[9,10] (Methods of Enzymatic Analysis, p. 651, 1963; Interpretive Enzymology, 1967). In the case of hepatitis the rise in serum glutamic oxaloacetate transaminase and glutamic pyruvate transaminase usually occurs before symptomatic changes in the bilirubin level and the onset of jaundice[9]. Recently it has been shown that the mean serum lactate dehydrogenase (LDH) activity of 11 volunteers with experimentally induced typhoid fever rose significantly within one day after the men had received a dose of typhoid organisms[11] (Metabolism 1971, In Press). Though this rise in LDH activity is not clinically significant when measured as total serum activity, it may become so when a sample of the serum is subjected to electrophoresis to reveal the isoenzyme pattern.

Serum alkaline phosphatase has been shown to increase in pneumococcal infection in mice[12] (Proc. Soc. Exp. Biol. Med. 124:812, 1967) and to decrease during virus injection in chickens[13] (Appl. Microbiol. 15: 677, 1967). Multiple forms of alkaline phosphatase activity have been demonstrated, the patterns of which fall into 3 rather distinct categories: one associated with hepatobiliary involvement; a second with parenchymal cell damage of the liver; and a third with increased osteoblastic activity[14] (Amer. J. Clin. Path. 40:349, 1963). Serum alkaline phosphatase acrylamide gel patterns may thus aid in diagnosis.

Polyacrylamide gel electrophoresis will be carried out using the Ortec model 4200 system. Quantitation will be achieved by analyzing either the gels themselves or photographic copies with the Farrand densitometer. Evaluation of the diagnostic potential of serum protein and isoenzyme patterns will be achieved by simultaneously analyzing the sera obtained from an individual at varius times during a controlled infection. Such serum samples will be provided by volunteer projects carried out at USAMRIID and by joint studies with contractors.

Our present efforts have been directed at determining the optimum conditions for electrophoresis and for assay of enzymic activities. An attempt is being made to develop a reliable quantitative measure of absolute quantities rather than relative values so as to be able to assess optimum conditions of storage for samples and to allow comparison between various infections.

Summary:

An evaluation of the suitability of electrophoresis of serum proteins and isoenzymes in an acrylamide gel medium as a diagnostic aid in infectious diseases was started recently. At present, efforts are directed towards determining optimal conditions for analysis.

Publications:

None

LITERATURE CITED

1. Banerjee, S., and J. N. Bhaduri. 1959. Serum protein-bound carbohydrates and lipids in cholera. Proc. Soc. Exp. Biol. Med. 101:340-341.

2. Sunderman, Jr., F. W. 1964. Studies of the serum proteins. VI. Recent advances in clinical interpretation of electrophoretic fractionations. Amer. J. Clin. Path. 42: 1-21.

3. Klainer, A. S., D. F. Clyde, P. J. Bartelloni, and W. R. Beisel. 1968. Serum glycoproteins in experimentally induced malaria in man. J. Lab. Clin. Med. 72:794-802.

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5. Gallin, J. I., D. Kaye, and W. M. O'Leary. 1969. Serum lipids in infection. New Eng. J. Med. 281:1081-1086.

6. Rollins, J. B., G. A. Burghen, and W. R. Beisel. 1970. The influence of altered viral virulence on the response of host serum $alpha_2$ glycoglobulins. J. Infect. Dis. 122:329-334.

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11.Wannemacher, R. W., H. L. DuPont, R. S. Pekarek, M. C. Powanda, R. B. Hornick, and W. R. Beisel. 1971. Sequential changes in individual serum amino acids, enzymes and trace metals following experimentally-induced typhoid fever in man. Metabolism, In press.

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ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 03:	Laboratory Identification of Biological Agents
Work Unit No. 096 03 402:	Development of Methods for the Detection and Assay of Interferon
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Division:	Virology
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Authors:	Bruno J. Luscri, Ph.D. (1) George W. Jordan, Major, MC (II)
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
	DA OL0855	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 01 06	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	03	402
b. CONTRIBUTING				
c. CONTRIBUTING	CDOG 1212b(9)			

11. TITLE (Precede with Security Classification Code)* (U) Development of Methods for Detection and Assay of Interferon for Rapid Identification of Illnesses of Military Importance (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS* 010100 Microbiology; 003500 Clinical Medicine; 004900 Defense

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
61 08	CONT	DA	C. In-house

17. CONTRACT GRANT

a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER*:
c. TYPE: NA d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.:

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	2	100
	CURRENT 72	2	100

19. RESPONSIBLE DOD ORGANIZATION

NAME:* USA Medical Research Institute of Infectious Diseases
ADDRESS:* Fort Detrick, Md 21701

RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION

NAME:* Virology Division USAMRIID
ADDRESS:* Fort Detrick, Md 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME:* Luscri, B. J.
TELEPHONE: 301 663-411 Ext 7241
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Jordan, G. W.
NAME:
DA

21. GENERAL USE

Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Interferon; (U) Viral interference; (U) Sindbis virus; (U) Semliki Forest virus; (U) Chikungunya virus; (U) Military Medicine

23. TECHNICAL OBJECTIVE.* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Develop tests for use in rapid diagnosis of viral diseases.

24 (U) An interferon of tissue culture origin will be produced and characterized. Standard methods for bioassay will be applied. Interferon is to be produced in quantity and partially purified. Antisera to partially purified interferon are to be tested for possible use in an immunological assay for interferon.

25 (U) 70 07 - 71 06 - I. Chick and mouse interferons have been produced and characterized. The mouse interferon possessed elevated antiviral activity with 50 micrograms or less of protein per milliliter. A carbohydrate present in the interferon fluids was also detected in L control fluids. The results of a fractionation procedure with an interferon preparation suggested that the carbohydrate was not associated with the bulk of the recovered interferon activity.

II. Computer techniques have been applied to the probit analysis of plaque reduction data. Rabbits and guinea pigs are currently being immunized with chick and mouse interferon in order to obtain antisera for possible use in an immunoassay.

Publications: Bact. Proc., p. 213, 214, 1971.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 03: Laboratory Identification of Biological Agents

Work Unit No. 096 03 402: Development of Methods for the Detection and Assay of Interferon

Description:

Develop methods for the detection and bioassay of interferon.

Progress, Part I:

Mouse L cell interferon was prepared (3 lots) according to the method previously described[1] (USAMRIID Annual Progress Report, FY 1969, p. 221). The preparations were assayed for interferon content by a plaque reduction method[2] (Virology 13:323, 1961), a cytopathic effect (CPE) inhibition method[3] (J. Nat. Cancer Inst. 38:771, 1967), and a hemagglutination yield reduction method[4] (Proc. Soc. Exp. Biol. Med. 135:340, 1970). The resulting interferon titers are shown in the first Table.

TABLE I. INTERFERON TITERS OF A MOUSE L CELL INTERFERON BY 3 METHODS OF BIOASSAY

	INTERFERON TITER (UNITS)		
CHALLENGE VIRUSES[a]	Plaque Reduction by 50%[b]	CPE Inhibition by 50%[c]	HA YIELD REDUCTION BY 0.5 LOG_{10}[c]
VSV	25,000	300	
GD7	25,000	200	32,000
EMC	1,600	100	
VEE	6,300		

a. Vesicular stomatitis, GD7, encephalomyocarditis, Venezuelan equine encephalomyelitis viruses.

b. Interferon units/3 ml.

c. Interferon units/ml

The antiviral activity of the processed L-NDV-B1 fluids was shown to be acid-stable, nondialyzable, trypsin sensitive, and nonsedimentable when centrifuged at 100,000 X g for 3 hr. In plaque reduction assays, linear dose response data were obtained, and the plaque size of the challenge viruses were reduced. The interferon fluids were not toxic for L cells or lethal for embryonating eggs, did not neutralize VSV directly, and demonstrated no peaks of maximum adsorption between the range of 210 to 400 nm. L cell control fluids did not contain an antiviral inducing property.

A carbohydrate reactive as fucose-bound protein[5/] (Methods of Biochemical Analysis, Vol I, p. 279.) was found in the interferon and L control fluids. Fractionation of an interferon fluid by precipitation at pH 11.6 with 0.1 N NaOH resulted in an alkali soluble fraction permitting 45% recovery of the antiviral activity (fraction B). Addition of 3 volumes of 95% ethanol to 1 volume of this supernate resulted in an alcohol precipitate with a 10.8% recovery of the antiviral activity (fraction C). A summary of this fractionation scheme with recovery of antiviral activity in the various fractions is presented in Table II.

TABLE II. SUMMARY OF FRACTIONATION PROCEDURE OF L-CELL INTERFERON AS BIOASSAYED AGAINST TRINIDAD VEE BY A PLAQUE REDUCTION METHOD

PREPARATION	INTERFERON RECOVERY FROM PREVIOUS STEP %
L Cell interferon	100
A Fraction (Ppt from pH 11.5)	0.2
B Fraction (supernate from pH 11.5)	45
C Fraction (Ppt from alcohol)	10.8
D Fraction (Supernate from alcohol)	0.0

The fractionation scheme suggested that the carbohydrate was not associated with interferon activity, and that antiviral activity could be obtained in fractions not reactive with the Lowry reagents used to detect protein.

Summary, Part I:

Chick and mouse interferons have been produced and characterized. The mouse interferon possessed elevated antiviral activity with 50 micrograms or less of protein per milliliter. A carbohydrate present in the interferon fluids was also detected in L control fluids. The results of a fractionation procedure with an interferon preparation suggested that the carbohydrate was not associated with the bulk of the recovered interferon activity.

Progress, Part II:

Currently, the interferon fluids are diluted 1:100 in maintenance medium, and stored at -60 C as a mouse interferon standard for use in our laboratory.

In order to obtain interferon preparations suitable for use as a laboratory standard and for use as an immunizing antigen several variables of the induction process were studied. For chick interferon Chikungunya and Rio Bravo viruses at a multiplicity of approximately one median suckling mouse intracerebral lethal dose per cell will result in a maximum interferon titer in 18 hr. Optimum conditions include ageing the chick cells for one week prior to induction. For mouse interferon, best yields were obtained by using L-cells aged for one week and inducing with Newcastle Disease virus (B_1 strain) at a multiplicity of approximately 100 median egg lethal doses per cell.

The interferons were shown to have the following properties: (1) induction process inhibited by actinomycin, (2) not sedimentable at 100,000 X g for 1 hr, (3) not dialyzable, (4) resistant to pH 2, (5) inhibits several unrelated viruses, (6) characteristic dose response, (7) activity destroyed by trypsin, (8) not active on cells of heterologous species. A laboratory standard of chick and mouse interferon has been prepared.

In choosing an interferon assay for routine use preliminary attempts were made with assays based on inhibition of CPE, inhibition of virus yield, inhibition in the yield of hemagglutinins, and plaque reduction. On the basis of overall convenience a plaque reduction method using vesicular stomatitis virus as the challenge agent has been adopted. For maximum sensitivity mouse interferon must be left in contact with L-cells for 24 hr while chick cells require 4-6 hr and show a decrease in sensitivity after prolonged contact with interferon. During the establishment of resistance the probit of developing plaques has been shown to be linearly related to the sum of the logarithms of time and concentration. Significant changes in the dose-response occur on ageing of cell monolayers to be used in a plaque reduction assay and for the most reproducible results chick cells should be used between days 1 and 3 and mouse cells between days 2 and 5 after planting. The data obtained from a plaque reduction assay are usually expressed as probits and the titer taken from a provisional line of a plot of probit on the logarithm of the interferon dilution. The weighting coefficient for use in this type of problem[b] (Ann. Appl. Biol. 36:196, 1949) has been used to modify a computer program for probit regression which has been used to calculate individual lines as well as to compare samples under the assumption of a common dose-response. A consideration of the weighting coefficient in this type of problem indicates that the interferon dose at which 27% of the plaques develop can be most precisely estimated and may allow greater discrimination between samples than the usual median plaque depressing dose (PDD_{50}) endpoint.

In order to obtain antisera to chick and mouse interferon for possible use in an immunoassay, rabbits and guinea pigs are currently being immunized.

Future plans include the application of an assay for human interferon to study the development of interferon in vaccinees.

Summary, Part II:

Computer techniques have been applied to the probit analysis of plaque reduction data. Rabbits and guinea pigs are currently being immunized with chick and mouse interferon in order to obtain antisera for possible use in an immunoassay.

Presentations:

1. Jordan, G. W. Application of probit analysis to interferon plaque reduction data. Presented at Annual Meeting of American Society for Microbiology, Minneapolis, Minn. 2-7 May 1971.

2. Luscri, B. J. Preparation, bioassay, and partial fractionation of an efficient L cell interferon. Presented at Annual Meeting of American Society for Microbiology, Minneapolis, Minn. 2-7 May 1971.

Publications:

1. Jordan, G. W. 1971. Application of probit analysis to interferon plaque reduction data. Bact. Proc. p. 214.

2. Luscri, B. J. 1971. Preparation, bioassay, and partial fractionation of an efficient L cell interferon. Bact. Proc. p. 213.

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2. Wagner, R. R. 1961. Biological studies of interferon. I. Suppression of cellular infection with Eastern equine encephalomyelitis virus. Virology 13:323-337.

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5. Winzler, R. J. 1955. Determination of serum glycoproteins. p. 279-311. In D. Glick (ed.), Methods of Biochemical Analysis, Vol. 2. Interscience Publishers, Inc., New York.

6. Wadley, F. M. 1949. Dosage-mortality correlation with number treated estimated from a parallel sample. Ann. Appl. Biol. 36: 196-202.

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ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 03:	Laboratory Identification of Biological Agents
Work Unit No. 096 03 403:	Separation, Purification and Concentration of Arbovirus Agents and Antigen-antibody Complexes
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Division:	Virology
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Author:	Neil H. Levitt, Captain, MSC
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

314

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION* DA OL0856	2. DATE OF SUMMARY* 71 07 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 01 06	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	03	403
b. CONTRIBUTING				
c. [illegible]	CDOG 1212b(9)			

11. TITLE (Precede with Security Classification Code)*
(U) Separation, Purification and Concentration of Arbovirus Agents and Antigen-Antibody Complexes for Military Medicine (11

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*
003500 Clinical Medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
61 11	CONT	DA	C. In-house

17. CONTRACT GRANT
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER:*
c. TYPE: NA d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	1	40
	CURRENT 72	1	100

19. RESPONSIBLE DOD ORGANIZATION
NAME:* USA Medical Research Institute of Infectious Diseases
ADDRESS:* Fort Detrick, Md 21701

RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
NAME:* Virology Division USAMRIID
ADDRESS:* Fort Detrick, Md 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME:* Levitt, N. H.
TELEPHONE: 301 663-4111 Ext 7241
SOCIAL SECURITY ACCOUNT NUMBER:

21. GENERAL USE
Foreign intelligence considered.

ASSOCIATE INVESTIGATORS
NAME:
NAME: DA

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Cellulose acetate electrophoresis; (U) Immunology; (U) Military Medicine; (U) Adenovirus; (U) Serology; (U) Antigen-antibody reactions

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Develop an invitro diagnostic test for rapid detection of antibody to viruses causing militarily significant disease.

24 (U) Virus-antibody complexes are separated and detected by cellulose acetate electrophoresis.

25 (U) 70 07 - 71 06 - An immunoelectrophoretic procedure utilizing microprecipitation on cellulose acetate plates has been developed for the laboratory detection of antibody to a specific virus. The usefulness of this technique in diagnosis was demonstrated with adenovirus-7 and homologous antiserum. Virus antigen was concentrated and partially purified by Genetron-113 extraction and banding on a cesium chloride cushion. Antibody to adenovirus-7 has been successfully detected with this technique in human serum specimens obtained from adenovirus-7 infected individuals. The electrophoretic res[illegible] ave thus far correlated with the presence of neutralizing antibody in serum.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 03: Laboratory Identification of Biological Agents

Work Unit No. 096 03 403: Separation, Purification and Concentration of Arobvirus Agents and Antigen-Antibody Complexes

Description:

Develop an in vitro diagnostic test for rapid detection of antibody causing militarily significant disease.

Progress:

The isolation and the identification of viruses, and the detection of increases in antibody concentration are the main tools used for the laboratory diagnosis of viral illnesses. Standard laboratory procedures are successfully employed for the majority of viruses commonly encountered in clinical cases, however, many of these procedures are tedious and require days to weeks for completion, while others can not be adapted for certain virus groups. More rapid immunochemical methods of detection are constantly being sought[1,2,3,4] (J. Immun. 98:314, 1967, 104:1031, 1970; Life Sci. 6:2135, 1967; Science 169:298, 1970).

A diagnostic test was developed in this laboratory for the rapid detection of serum antibody and/or viral antigen in clinical specimens. The test is composed of two steps: microprecipitation of virus-antibody complexes and the detection of these complexes by cellulose acetate electrophoresis. More specifically, microquantities (50 µl) of a concentrated virus preparation are reacted with similar amounts of serum dilutions at 37 C for 1 hr or 4 C overnight in microtiter plates. Ten microliters of each virus-serum mixture is applied to a 1 X 3 inch cellulose acetate plate and electrophoresed for 15 min at 220 V in a chamber containing trisbarbital buffer (pH 8.4, ionic strength 0.05). The plates are stained with Ponceau S stain, rinsed in 2% acetic acid to remove excess stain, air-dried and then examined for elliptical precipitates at the site of sample application. A sample of virus and serum are each mixed individually with saline and tested concurrently with the virus-serum mixtures as controls. A visible precipitate, resulting from the interaction of virus and serum together with negative antigen and serum controls, indicates the presence of specific antibody.

A previous report[5] (USAMRIID Annual Progress Report, FY 1970, p. 235) described the development and refinement of this technique with a model virus system of tobacco mosaic virus and homologous rabbit antiserum. Recently, we have been successful in adapting this procedure to an animal virus system employing adenovirus, type 7 as test antigen. The virus was grown in roller bottles of KB cells, concentrated and partially purified using a modification of the procedure described by Green and Pina[6] (Virology 20:199, 1963). Approximately 20 hr

postinfection, the cells were freeze-thawed 3 X to release intracellular virus; the mixture was centrifuged at low speed to sediment the cellular debris. The fluid phase was extracted several times with Genetron-113 to remove much of the cellular contamination; the virus was then banded by high speed centrifugation onto a CsCl cushion (density = 1.59). After dialysis overnight to remove the CsCl, 1% human serum albumin was added to stabilize the antigenicity of the purified virus preparation. Antigenic potency was determined by the reaction of antigen and hyperimmune rabbit serum.

Preliminary experiments employing adenovirus antigen and clinical specimens demonstrated the existence of nonmigrating proteins in human sera that resulted in false positive reactions; that is, a precipitate was visible at the origin with samples receiving no antigen (serum control). This observation was not seen with any of the animal sera previously tested. We devised a rapid and efficient procedure for the removal of the nonmigrating proteins from human serum. A serum aliquot was extracted for 30 sec with 2-4 volumes of Genetron-113, centrifuged at low speed to separate aqueous and organic phases and the top aqueous phase (serum) was removed by aspiration. When tested with adenovirus-7 antigen in the electrophoretic technique, both human and rabbit sera showed no loss in antibody titer.

In an attempt to correlate electrophoretic results with a standard serological test, 20 human sera previously tested for neutralizing and complement fixing antibody at WRAIR were screened using our electrophoretic procedure. Data from this experiment can be seen in Table I.

A positive reaction on cellulose acetate plates was observed with those sera having a strong neutralization titer. No correlation, however, was seen with complement fixation (CF) titers. Statistical correlation between electrophoretic and neutralization titers awaits the availability of additional clinical specimens.

TABLE I. CORRELATION OF RESULTS OBTAINED FROM ELECTROPHORETIC, NEUTRALIZATION AND COMPLEMENT FIXATION TESTS ON 20 SERA[a] USING ADENOVIRUS TYPE 7 AS ANTIGEN

ELECTROPHORETIC REACTION[b]	RECIPROCAL TITER[c]	
	Neutralization	CF
POSITIVE	≥256	160
	256	80
	256	10
	≥128	10
	≥128	<5
	128	20
	64	≥160
	64	80
	64	80
NEGATIVE	<4	80
	<4	20
	"	20
	"	10
	"	10
	"	<5
	"	<5
	"	<5
	"	<5
	"	<5

a. No electrophoretic test performed on one specimen.

b. Screened at 1:10 dilution of serum

c. Performed at WRAIR

Summary:

An immunoelectrophoretic procedure utilizing microprecipitation on cellulose acetate plates has been developed for the laboratory detection of antibody to a specific virus. The usefulness of this technique in diagnosis was demonstrated with adenovirus, type 7, and homologous antiserum. Virus antigen was concentrated and partially purified by Genetron-113 extraction and subsequent banding on a CsCl cushion. Antibody to adenovirus has been successfully detected with this technique in human serum specimens obtained from adenovirus infected individuals. The electrophoretic results have thus far correlated with the presence in serum of neutralizing antibody.

Publications:

None

LITERATURE CITED

1. Cuadrado, R. R., J. Casals. 1967. Differentiation of arboviruses by immunoelectrophoresis. J. Immun. 98:314-320.

2. Gocke, D. J., and C. Howe. 1970. Rapid detection of Australia antigen by counterimmunoelectrophoresis. J. Immun. 104:1031-1032.

3. Neurath, A. R., B. A. Rubin, M. Z. Bierly, and S. Wong. 1967. Titration of antibodies in sera by an indirect quantitative fluorescent precipitin test. Life Sci. 6:2135-2141.

4. Saravis, C. A., C. Trey, and G. F. Grady. 1970. Rapid screening test for detecting hepatitis-associated antigen. Science 169:298-299.

5. U. S. Army Medical Research Institute of Infectious Diseases. 1 July 1970. Annual Progress Report, FY 1970, p. 235 to 239. Fort Detrick, Md.

6. Green, M., and M. Pina. 1963. Biochemical studies on adenovirus multiplication. IV. Isolation, purification, and chemical analysis of adenovirus. Virology 20:199-207.

ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defe .se Aspects of Biological Agents (U)
Task No. 1B662711A096 03:	Laboratory Identification of Biological Agents
Work Unit No. 096 03 404:	Use of Antiglobulin for Early Detection of Arbovirus Antibody
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Division:	Virology
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Author:	Albert T. McManus, Captain, MSC
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
	DA OL0904	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
70 12 22	D. CHANCE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	03	404
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	CDOG 1212b(9);	1412a(2)		

11. TITLE (Precede with Security Classification Code)* (U) Use of Antiglobulin for Early Detection of Arbovirus Antibody of Diseases of Military Importance (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*
010100 Microbiology; 003500 Clinical Medicine; 004900 Defense

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
70 12	CONT	DA	C. In-house

17. CONTRACT GRANT
a. DATES/EFFECTIVE — EXPIRATION
b. NUMBER*
c. TYPE NA — d. AMOUNT
e. KIND OF AWARD — f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	1	8
	CURRENT 72	1	78

19. RESPONSIBLE DOD ORGANIZATION
NAME* USA Medical Research Institute of Infectious Diseases
ADDRESS* Fort Detrick, Md 21701
RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
NAME* Virology Division
ADDRESS:* USAMRIID
Fort Detrick, Md 21701
PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME:* McManus, A. T.
TELEPHONE 301 663-4111 Ext 7241
SOCIAL SECURITY ACCOUNT NUMBER
ASSOCIATE INVESTIGATORS
NAME:
NAME: DA

21. GENERAL USE
Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Arboviruses; (U) Antigen-antibody reactions; (U) Serology; (U) Human volunteer; (U) Military medicine

23. TECHNICAL OBJECTIVE.* 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Attempt to hasten identification of an arbovirus infection in man.

24 (U) Investigations will involve the use of antiglobulin neutralization in a plaque reduction test.

25 (U) 70 12 - 71 06 - A constant virus varying antiserum concentration plaque reduction technique has been shown effective for detection of antibody to the live, attenuated Venezuelan equine encephalomyelitis vaccine strain. This technique is presently being adapted to 17-D and Asibi strains of yellow fever virus using sera from volunteers immunized with the standard 17-D vaccine.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 03: Laboratory Identification of Biological Agents

Work Unit No. 096 03 404: Use of Antiglobulin for Early Detection of Arbovirus Antibody

Description:

Attempt to hasten identification of an arbovirus infection in man.

Progress:

A pilot project to establish a plaque reduction neutralization technique has been completed. A constant virus-varying-antiserum concentration method has been shown effective to detect antibody levels in an individual immunized with Venezuelan equine encephalomyelitis vaccine, Live, Attenuated. No prevaccination serum was available for this individual. The titer of the individual was calculated by probit paper plotting with a 50% plaque reduction used as an endpoint. The titer of 3.5 logs of neutralizing antibody was repeatable. Computer analysis of one of the titrations yielded titers of 3.47 logs and 3.60 logs using 2 regression analysis techniques.

Application of the technique to human sera drawn early was the next phase of the project. A coordinated project was initiated with Medical Division Project No. FY 71-3, "Evaluation of Human Metabolic Responses to 17-D Strain of Yellow Fever Vaccine". Sera was obtained from individuals on this project at daily or alternate day intervals for days -3, 0, 1-10, and 28. Total nonspecific levels of IgG, IgM, IgA and C'_3 complement during the 10-day postinfection period were calculated by immunoradial diffusion for 6 individuals. This data is presently being analyzed.

Plaque reduction analysis is complicated by slow plaque formation with 17-D virus and the presence of viremia in infected subjects. Determination of the duration and amount of plaque forming virus in serum of human subjects is in progress. The 17-D strain has been shown to produce plaques using chick embryo fibroblast, duck embryo fibroblast and baby hamster kidney (BHK-21) cells. Chick and BHK-21 cells require 9.5 days after overlay to develop plaques, duck cells require 5 days. 17-D strain has not produced plaques in the VERO cell line, although the virulent strain of yellow fever, Asibi Strain will. The differential growth will be used to distinguish plaque type in the plaque reduction Assay.

Upon establishment of the earliest date postinfection that neutralizing antibody can be detected, multispecific and monospecific antisera to human immunoglobulins will be added to virus human serum mixtures. It is believed that this method will neutralize infective virus-antibody complexes. Demonstration of infective virus-antibody complexes will give qualitative and quantitative evidence of the immune response to 17-D infection in man.

Summary:

A constant virus-varying-antiserum concentration plaque reduction technique has been shown effective for detection of antibody to VEE vaccine. This technique is presently being adapted to 17-D and Asibi strains of yellow fever virus using sera from 17-D strain immunized volunteers.

Publications:

None.

ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 03:	Laboratory Identification of Biological Agents
Work Unit No. 096 03 800:	Immunological Studies with Microbial Toxins
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Divisions:	Bacteriology and Animal Assessment
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Authors:	Virginia G. McGann, Ph.D. (I, II) Richard O. Spertzel, Lt. Colonel, VC (I, II) Joseph C. Denniston, Captain, VC (II) Douglas W. Mason, Captain, VC (I) Glen E. Marrs, Jr., Captain, VC (I) Elizabeth O. Roberts, Ph.D. (I, II)
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

324

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
	DA OL0876	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 01 06	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	03	800
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	CDOG 1212b(9); 1412a(2)			

11. TITLE (Precede with Security Classification Code)*
(U) Immunological Studies with Microbial Toxins of Military Medical Importance (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*
016800 Toxicology; 003500 Clinical Medicine; 010100 Microbiology; 004900 Defense

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
66 10	CONT	DA	C. In-house

17. CONTRACT GRANT
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER:*
c. TYPE: NA d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	3	50
	CURRENT 72	3	80

19. RESPONSIBLE DOD ORGANIZATION
NAME:* USA Medical Research Institute of Infectious Diseases
ADDRESS:* Fort Detrick, Md 21701
RESPONSIBLE INDIVIDUAL
NAME: Crozier, D.
TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION
NAME:* Bacteriology Division USAMRIID
ADDRESS:* Fort Detrick, Md 21701
PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME:* McGann, V. G.
TELEPHONE: 301 663-4111 Ext 7341
SOCIAL SECURITY ACCOUNT NUMBER:
ASSOCIATE INVESTIGATORS
NAME: Spertzel, R. O.
NAME: Roberts, E. O. DA

21. GENERAL USE
Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Enterotoxin, staphylococcal; (U) Toxoid; (U) Immunology; (U) Antibodies; (U) Hypersensitivity; (U) Military Medicine

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Investigate immunologic responses of a susceptible host after exposure to microbial toxin and after immunization with toxoid.

24 (U) Investigate serological activity of staphylococcal enterotoxins and their toxoids; evaluate the role of antibody in resistance to ingested toxin, and define the pathogenesis of acute response following reexposure to toxin.

25 (U) 70 07 - 71 06 - Hyperimmune sera produced with types A, B or C staphylococcal enterotoxins contained minor antibodies for each of the other toxins. Surveys for naturally-acquired antibody indicated that incidence and multiplicity of antibodies in human sera were significantly higher than in monkey sera (p less than 0.001). Unlike resistance to parenteral exposure, resistance to ingested toxin was not directly related to humoral antibody. The findings suggest that the primary immunogenic response to ingested toxin may involve local production of secretory antibody, and that repeated exposures with relatively large amounts of toxin are required to stimulate the humoral antibody system. In parenterally exposed or toxoid-immunized monkeys, however, resistance to subsequent ingestion of toxin appeared to be associated with humoral antibody titer.

Gel immunoprecipitation studies suggested that components of a trivalent toxoid for types A, B and C had common antigens as well as type-specific antigens.

Pathophysiological and immunological studies demonstrated that type II hypersensitivity was responsible for the acute reponse of monkeys to reexposure with type B toxin.

Publication: J. Infect. Dis., 1971, In press.

*Available to contractors upon originator's approval

DD FORM 1498 MAR 68 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 03: Laboratory Identification of Biological Agents

Work Unit No. 096 03 800: Immunological Studies with Microbial Toxins

Description:

Investigate immunologic responses of a susceptible host after exposure to microbial toxin and after immunization with toxoid.

Progress, Part I:

In previous studies[1, 2] (USAMRIID Annual Progress Report, FY 1970, p. 241; J. Infect. Dis. In press, 1971) serologic techniques were evaluated for their efficiency in estimating resistance of monkeys to parenteral exposure with staphylococcal enterotoxin B (SEB). Low-titering antibodies, detectable by hemagglutination (HA), were associated with resistance to intravenous (IV) challenge with $\leq$25 median illness doses (ED_{50}), whereas SEB-precipitating antibody was required for resistance to higher doses. Essentially no information was available for evaluation of resistance to ingested toxin or of the immunologic response to type A (SEA) and type C (SEC) enterotoxins. Consequently, studies were designed to investigate the role of antibody in resistance to ingestion of SEB, and, when limited quantities of purified SEA and SEC became available, to examine the serological activity of these toxins.

Enterotoxins A and C. Purified SEA and its homologous antisera were prepared in the Biological Laboratories, Fort Detrick, and purified SEC, by Pathology Division, USAMRIID. Serologic techniques developed for SEB appeared to be equally satisfactory for SEA studies. In HA titrations, however, the reactions of SEC appeared to be atypical. Sheep erythrocytes (RBC) coupled to freshly reconstituted SEC, agglutinated to abnormally high HA titers; moreover, delayed lysis of agglutinated RBC was observed. Typical staphylococcal hemolysins were not responsible for the lytic activity because lysis never occurred in the absence of antibody. By employing block titrations with decreasing quantities of SEC, it was possible to eliminate lytic effects, as well as unusually high titers in sera from nonimmune monkeys, without affecting HA reactions in specific antisera. This atypical activity was also destroyed during storage of reconstituted SEC for 2 weeks at 4 C.

Hyperimmune sera for any 1 of the 3 enterotoxins showed minor reactions with each of the other types (Table I). Cross-reacting precipitins for types A and B were difficult to demonstrate, but precipitin reactions with SEC were observed in almost all sera. SEC precipitation showed partial identity with that of homologous antigens.

TABLE I. ANTIBODIES FOR HOMOLOGOUS AND HETEROLOGOUS TYPES OF STAPHYLOCOCCAL ENTEROTOXIN IN SINGLE FACTOR HYPERIMMUNE SERA.

TEST ANTIGEN	RECIPROCAL HA TITER AND PRECIPITIN REACTION					
	Anti-SEA (Rabbit 80)		Anti-SEB (Goat 222)		Anti-SEC (Goat 406)	
	HA	Precipitin	HA	Precipitin	HA	Precipitin
SEA	160,000	4+	80	Neg	10	Neg
SEB	320	Neg	20,000	4+	640	$\pm$
SEC	80	$\pm$	640	$\pm$	40,000	4+

Naturally-acquired antibodies for the 3 enterotoxins were detected in human and monkey sera (Table II). Approximately 80% of human sera, obtained from a group of institutionalized adult males, had antibody for the 3 enterotoxins, whereas over 60% of monkey sera had no detectable antibody and only 6% had antibody for more than 1 type. SEC antibodies were the predominant type in monkey sera. Although mean HA titers were low, individual titers of 1:2560 occasionally were found for each of the 3 types. Absorption studies indicated that, even in sera with low titers, toxin-antitoxin reactions were specific for each type. The incidence of naturally-acquired precipitating antibody was considerably lower than that of HA antibody. Only 65% of human sera contained precipitins and only 8% reacted with all enterotoxins; SEA precipitins had the lowest incidence.

Toxoids. Reactions of a trivalent toxoid for SEA, SEB and SEC (prepared in Pathology Division, USAMRIID) were compared with those of monovalent type B toxoid, Lot #87825. Precipitin reactions were demonstrated between the trivalent preparation and minor antibody components in hyperimmune sera produced against each of the 3 enterotoxins; monovalent type B toxoid reacted primarily with a minor antibody in SEB antitoxins. Unlike the reactions of single-factor antitoxins with the 3 types of toxin, reactions of these sera with trivalent toxoid appeared to involve identical, or closely related, antibodies. Tests for identity between reactions of monovalent type B toxoid with specific antitoxoid and trivalent toxoid with antitoxins, however, indicated a high degree of type specificity. Monovalent SEA and SEC toxoids and their antisera will be necessary for a more detailed analysis of toxoid serology.

TABLE II. HEMAGGLUTINATING ANTIBODIES FOR STAPHYLOCOCCAL ENTEROTOXINS, SEA, SEB AND SEC, IN 132 HUMAN AND 213 MONKEY SERA.

REACTION GROUP	HUMAN SERA % Reactors	Titer[a/] SEA	SEB	SEC	MONKEY SERA % Reactors	Titer[a/] SEA	SEB	SEC
No detectable antibody	1	-	-	-	63	-	-	-
SEA	2	28	-	-	2	68	-	-
SEB	2	-	25	-	5	-	80	-
SEC	2	-	-	40	24	-	-	53
SEA + SEB	3	13	20	-	0	-	-	-
SEA + SEC	5	50	-	64	3	59	-	39
SEB + SEC	6	-	87	57	3	-	57	16
SEA + SEB + SEC	80	64	126	130	0	-	-	-

a. Geometric mean reciprocal titer.

The antibody response of monkeys to each component in the trivalent toxoid was comparable to that seen previously following injection of monovalent SEB toxoid.[1/] After one injection of toxoid, monkeys with naturally-acquired antibody for 1 or more toxins exhibited a booster response of the corresponding antibody. In the absence of preexisting antibody, antitoxin titers developed slowly.

Intragastric (IG) challenge. Preliminary investigations on response of monkeys to challenge with SEB by gavage have been reported elsewhere[2/] (J. Infect. Dis. In press, 1971). Unlike our findings with parenteral routes of exposure, resistance to IG challenge was unrelated to naturally-acquired humoral antibody, and SEB could not be detected in serum at any time. In monkeys with preexisting antibody, however, a progressive decrease in serum antibody occurred for at least 6 hr after challenge, possibly suggesting extravascular localization and combination with toxin. Few monkeys that lacked naturally-acquired antibody developed titers following Ig challenge; however, in those that did, maximum titers were achieved within 2 weeks, suggestive of a secondary response.

Prior experience with SEB given by the parenteral route or prior immunization with toxoid appeared to be effective in producing protection against challenge by the IG route. In such monkeys resistance appeared to be related to the presence of humoral antibody (Table III).

TABLE III. THE EFFECT OF PRIOR IMMUNIZATION WITH TOXOID OR INTRAVENOUS CHALLENGE WITH TOXIN ON RESPONSE OF MONKEYS TO CHALLENGE BY GAVAGE.

CHALLENGE DOSE ED_{50}[a]	TREATMENT	NO. ILL/NO. TESTED BY PRECHALLENGE HA TITER	
		≤1:80	>1:80
10	Toxoid immunized	9/11	1/5
	Prior experience	1/1	1/5
	None	6/7	ND[b]
100	Prior experience	ND	1/6
	None	4/4	ND

a. Median illness doses, administered by gavage.

b. Not done.

Reexposure studies were initiated to allow a more detailed examination of response to IG challenge. In the first study, a group of monkeys that had naturally-acquired antibody at time of arrival in these laboratories was challenged with 1-100 IG ED_{50} at 4-mon intervals. At the time of primary exposure, most monkeys had decreased antibody titers and some were antibody-negative. In the second study monkeys that lacked naturally-acquired antibody were challenged at more frequent intervals with 1-10 IG ED_{50}.

In contrast to antibodies acquired by parenteral exposure or immunization, naturally-acquired humoral antibody was unrelated to resistance to the primary exposure; such antibodies were associated with rapid development of resistance to subsequent exposures (Table IV).

TABLE IV. RELATIONSHIP OF SEQUENTIAL EXPOSURES AND HUMORAL ANTIBODY ON RESPONSE OF MONKEYS TO CHALLENGE BY GAVAGE.

CHALLENGE	PRECHALLENGE HA TITER	NUMBER ILL/NUMBER TESTED		
		Challenge Dose (ED_{50})[a]		
		1	10	100
1 - 0 wk	≤1:80	23/27*	23/27	11/11
	>1:80	2/8	3/4	8/8
2 - 16 wk	≤1:80	8/12	17/24	11/11
	>1:80		1/5***	4/8*
3 - 32 wk	≤1:80	3/6	8/17**	5/9*
	>1:80		2/12***	5/9*
Antibody-negative control		10/17	17/19	7/7

a. Median illness dose, by gavage.

* Response significantly different from that of corresponding control monkeys: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

It was of interest to note that monkeys with low titers were less resistant than antibody-negative control monkeys to primary challenge with 1 ED_{50} ($p < 0.05$). Unlike our previous findings with parenteral challenges. conversion or increase of HA titer rarely occurred; HA-positive monkeys with titers of >1:80 often developed precipitins. Although antibody titers were not effective as guides for predicting resistance of individuals to IG exposure, high titers generally were associated with delayed onset and fewer episodes of illness, suggesting partial resistance.

Monkeys with no naturally-acquired antibody became resistant to IG challenge after 4 sequential exposures with 1 or 10 IG ED_{50} (Table V). Resistance developed before 50% of the monkeys had detectable levels of humoral antibody. Moreover, no signs of illness were observed following subsequent challenge with 10-100 IG ED_{50}. These findings suggest that if antibody is associated with resistance to ingested toxin, it probably is localized in or near the gut.

TABLE V. ILLNESS AND ANTIBODY RESPONSE OF MONKEYS TO SEQUENTIAL CHALLENGES WITH ENTEROTOXIN B ADMINISTERED BY GAVAGE (6 MONKEYS/GROUP).

CHALLENGE		CHALLENGE DOSE (ED_{50})[a/] 1		10	
No.	Week	No. Ill	No. Antibody-Positive	No. Ill	No. Antibody-Positive
1	0	4	0	5	0
2	16	4	0	6	0
3	22	4	0	5	1
4	24	1	0	1	3
5	26	0	1	0	3
6	28	0	3	0	4

a. Median illness dose

Summary, Part I:

The serological activity of type A and type C enterotoxins and factors involved in resistance to ingestion of type B enterotoxin were investigated. Hyperimmune sera produced with types A, B or C contained minor antibody components that reacted with each of the other toxins. Surveys for naturally-acquired antibody revealed that essentially all human sera reacted with 1 or more types and 80% had antibody for the 3 enterotoxins, whereas over 60% of monkey sera had no detectable antibodies and only 6% had antibody for more than 1 type of toxin. Antibodies for type C were found most frequently. Gel precipitin reactions with trivalent toxoid for types A, B and C suggested that the component toxoids had closely related antigens; tests for identity, however, with a monovalent type B toxoid indicated a high degree of antigen specificity.

Unlike resistance to parenteral exposure, resistance to primary exposure with ingested toxin was unrelated to the presence of naturally-acquired humoral antibody. Monkeys with such antibody, however, rapidly developed resistance to subsequent exposures. When antibody-negative monkeys were challenged repeatedly with enterotoxin B administered by gavage, resistance developed before humoral antibodies were detected, suggesting that the immunogenic response may involve local production of secretory antibody.

Progress and Summary, Part II:

Investigations were conducted with previously-exposed and toxoid-immunized monkeys to define the pathogenesis of acute illness and death following intravenous challenge with SEB. Results of these studies have been reported[3] (CES Annual Report, FY 1970, p. 261). In brief, manifestations of acute response were differentiated from the typical enterotoxic syndrome and were consistent with hypersensitivity reactions in subhuman primates. Cutaneous sensitivity and serological studies implicated a type II response; reactions occurred at *in vivo* toxin:antibody ratios of 1:1 - 2:1.

Publications:

1. McGann, V. G., J. B. Rollins, and D. W. Mason. 1971. Evaluation of resistance to staphylococcal enterotoxin B. Naturally-acquired antibodies of man and monkey. J. Infect. Dis. In press.

LITERATURE CITED

1. U. S. Army Medical Research Institute of Infectious Diseases. 1 July 1970. Annual Progress Report, FY 1970. p. 241 to 252. Fort Detrick, Maryland.

2. McGann, V. G., J. B. Rollins, and D. W. Mason. 1971. Evaluation of resistance to staphylococcal enterotoxin B. Naturally-acquired antibodies of man and monkey. J. Infect. Dis. In press.

3. Commission on Epidemiological Survey. December 1970. Annual Report to the Armed Forces Epidemiological Board, FY 1970. p. 261 to 274. Fort Detrick, Maryland.

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ANNUAL PROGRESS REPORT

Project No. 1B662711A096:	Medical Defense Aspects of Biological Agents (U)
Task No. 1B662711A096 03:	Laboratory Identification of Biological Agents
Work Unit No. 096 03 801:	Radioimmunological Assay of Staphylococcal Toxins and Other Exoproteins
Reporting Installation:	U. S. Army Medical Research Institute of Infectious Diseases Fort Detrick, Maryland
Division:	Pathology
Period Covered by Report:	1 July 1970 to 30 June 1971
Professional Authors:	William S. Collins, II, Lt Colonel, MSC (I) Donald E. Kahn, Captain, VC (II)
Reports Control Symbol:	RCS-MEDDH-288(R1)
Security Classification:	UNCLASSIFIED

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
				DA OL0902	71 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
71 01 06	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62711A	1B662711A096	03	801
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	CDOG 1212b(9)	1412a(2)		

11. TITLE (Precede with Security Classification Code)*

(U) Radioimmunological Assay of Staphylococcal Toxins and Other Exoproteins Causing Diseases of Military Importance (11)

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

003500 Clinical Medicine; 004900 Defense; 016800 Toxicology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
70 07	CONT	DA	C. In-house

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:* NA

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.:

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 71	2	50
	CURRENT 72	2	61

19. RESPONSIBLE DOD ORGANIZATION

NAME:* USA Medical Research Institute of Infectious Diseases

ADDRESS:* Fort Detrick, Md 21701

RESPONSIBLE INDIVIDUAL

NAME: Crozier, D.

TELEPHONE: 301 663-4111 Ext 5233

20. PERFORMING ORGANIZATION

NAME:* Pathology & Animal Assessment Div USAMRIID

ADDRESS:* Fort Detrick, Md 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME:* Collins, II, W. S.

TELEPHONE: 301 663-4111 Ext 6206

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Kahn, D. E.

NAME: DA

21. GENERAL USE

Foreign intelligence considered.

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Toxins; (U) Radioimmunoassay; (U) Exoproteins; (U) Staphylococcus; (U) Military medicine

23. TECHNICAL OBJECTIVE.* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) Develop a radioimmunoassay capable of detecting rapidly and quantitating small amounts of staphylococcal toxins and other exoproteins.

24 (U) Liquid- and solid-state techniques for radioimmunoassay are applied to develop a rapid test for detection and quantitation of toxins.

25 (U) 70 07 - 71 06 - I: A solid phase radioimmunological assay system has been developed to assay staphylococcal enterotoxin B (SEB) in body fluids, broths, and purified form. Brome acetyl acetate (BAC) is used to bind labeled SEB antibody to cellulose particles. After reacting, a gamma counter is used. This method is reliable and accurate down to levels of 0.01 microgram per milliliter.

Preliminary work has begun on the assay of SEA, SEC, and other exoproteins. The preparation of BAC and SEA and BAC and SEC is completed and radiolabeling of the antigens is in progress.

II. A double-antibody radioimmunoassay was adapted for use with SEB. This is a liquid-state test. Preliminary tests show that the method is satisfactory for detecting as little as 0.01 microgram per milliliter.

Comparison testing of the 2 methods is necessary since both have greater sensitivity than the usual immunodiffusion test.

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 1B662711A096: Medical Defense Aspects of Biological Agents (U)

Task No. 1B662711A096 03: Laboratory Identification of Biological Agents

Work Unit No. 096 03 801: Radioimmunological Assay of Staphylococcal Toxins and Other Exoproteins

Description:

Develop a radioimmunoassay capable of detecting rapidly and quantitating small amounts of staphylococcal toxins and other exoproteins.

Progress, Part I:

A method has been developed to assay staphylococcal enterotoxin B (SEB) in body fluids, broths, etc., sensitive to 0.01 μg/ml, approximately 2 logs more sensitive than immunodiffusion methods. Goat anti-SEB is attached to Brome acetyl cellulose to provide a carrier particle. This antibody-cellulose particle is then incubated with labeled ^{125}SEB and graduated amounts of natural SEB in a competitive inhibition system. After 15 min at room temperature and 2 hr at 4 C, both with agitation, the antigen-antibody complex is centrifuged at 15,000 rpm for 15 min. One ml of the supernatant is then removed and counted in a gamma counter. The counts are compared to a standard curve set up in the same manner with known amounts of antigen; the values are determined for the unknowns from this curve. Multiple examinations have been made and compared to immunodiffusion assays done by Bacteriology Division. The solid phase radioimmunological assay for SEB compares favorably with immunodiffusion down to about 1.0 μg/ml of SEB where immunodiffusion stops being useful. The radioimmunological assay appears to be usable, accurate, and reliable beyond the range of immunodiffusion, to levels of $\leq$0.01 μg/ml SEB. This is a distinct improvement over conventional methods both in sensitivity and in time.

Another factor not considered when the project was begun was the assay of antibody in serum. When examining immune serum for SEB it was found that the radioimmunological assay apparently gave an antibody assay which could be expressed as μg/ml equivalent of SEB. This was examined on numerous immune specimens and found to correlate well with hemagglutination (HA) titers. Further studies are in progress on this phase of the system as it would appear to be an extremely sensitive method for measuring antibody.

Summary, Part I:

A solid phase radioimmunological assay method has been developed for the measurement of SEB and SEB antibody. Further work is in progress on SEA, SEC, and other exoproteins.

Progress, Part II:

A double-antibody radioimmunoassay for SEB has been developed, using the following reagents:

1. Diluent: 0.05 M barbital buffer, pH 8.6, containing 0.1% gelatin.

2. SEB: Lot 14-30. Diluted to a working concentration of 2 μg/ml in normal monkey serum. Further dilution is made with barbital-gelatin to obtain SEB concentrations of 500, 250, 125, 62, 31, 16, 8, 4, and 2 ng/ml for use in constructing a standard curve.

3. ^{125}I-SEB: Lot 14-30 SEB is coupled to ^{125}I by chloramine-T oxidation[1/] (Biochem. J. 89:114, 1963). Much of the ^{125}I-SEB used in developing the assay has been supplied by Pathology Division, USAMRIID.

4. Rabbit Anti-SEB Serum: Obtained from male New Zealand white rabbits hyperimmunized by multiple inoculations of Lot 14-30 SEB in Freund's complete adjuvant.

5. Goat Anti-Rabbit Serum: Obtained from goats administered whole rabbit serum in Freund's complete adjuvant.

For each lot of ^{125}I-SEB prepared, it is necessary to determine the working concentration of labeled SEB to be employed in the test. By convention, the dilution of ^{125}I-SEB that produces 2,000 counts/5 min of radioactivity in the precipitate formed by mixing 100 μl ^{125}I-SEB, 100 μl of SEB (500 ng/ml), 500 μl of undiluted rabbit anti-SEB serum, and 100 μl of goat anti-rabbit serum.

The working dilution of rabbit anti-SEB serum used in the assay is one that binds 50-66% of the standard amount of ^{125}I-SEB in the absence of unlabeled SEB.

The immunoassay consists of adding 100 μl volumes of solutions to be tested for SEB, or of solutions containing known concentrations of SEB (for use in constructing a standard curve) to anti-SEB serum, ^{125}I-SEB, and a goat anti-rabbit serum. The precipitates are compacted by centrifugation and counted for radioactivity. All determinations are made on 3 replicate tubes. The optimal protocol is:

1. Place 500 μl rabbit anti-SEB (diluted to working concentration in barbital-gelatin buffer) in test tubes (12 x 75 mm) to be used for standard curve determinations, and for the assay of test materials for SEB ("unknowns").

2. Place 350 μl of diluent in 3 tubes to be used as antiserum controls.

3. Add 100 μl of appropriate reagents to standard tubes and unknowns. 100 μl of diluent is added to tubes designated as 0 ng/ml standards.

4. Incubate mixtures 5 hr at 5 C.

5. Add 100 μl of the working concentration of ^{125}I-SEB to all tubes. Put 100 μl of ^{125}I-SEB in tube for total count determination.

6. Incubate at 5 C for 16 hr.

7. Add 100 μl of normal rabbit serum (diluted 1:5 in barbital-gelatin) to standard tubes and unknowns. Add 350 μl of the normal rabbit serum to the antiserum blanks.

8. Add 100 μl of goat anti-rabbit serum (diluted 1:5) to each tube; mix.

9. Incubate 24 hr at 5 C.

10. Centrifuge at 2,500 rpm (International PR-2 Centrifuge), 5 C for 3 hr.

11. Discard supernatant fluid and place tubes in scintillation tubes for counting.

12. Construct a standard curve from samples containing known amounts of SEB.

13. Calculate concentrations of SEB in unknowns from the standard curve.

This radioimmunoassay method developed has proven to be 100-fold more sensitive than the immunodiffusion technique with which it was compared. The 2 tests were consistent when μg amounts of toxin were assayed (Table I).

TABLE I. COMPARISON OF RADIOIMMUNOASSAY AND IMMUNODIFFUSION TECHNIQUES IN A NONEXPERIENCED MONKEY GIVEN 1 mg/kg SEB IV

TIME	RADIO-ASSAY (μg/ml)	IMMUNODIFFUSION
Pre-exposure	ND[a/]	0
2 min	12.20	11.2
15 min	4.00	2.8
30 min	0.91	1.0
1 hr	0.09	0
2 hr	0.02	0
4, 6, 8, 10, 18, 24, 30, 34, 48 hr	ND	Not tested
49 hr (terminal)	0.01	0

a. ND = Not detectable.

Values obtained by radioimmunoassay have been reproducible in multiple determinations. Data from a series of standard tubes are included in Table II.

TABLE II. THE COMPETITIVE EFFECT ON ^{125}I-SEB BINDING WITH ANTI-SEB SERUM CAUSED BY THE ADDITION OF UNLABELED SEB

SEB ng/ml	% ^{125}I-SEB BOUND
0	66.8
1	66.4
2	66.3
4	65.4
8	64.4
16	62.4
31	56.5
62	48.9
125	36.6
250	20.1
500	8.0
1000	0.1

Summary, Part II:

The double-antibody radioimmunoassay technique was adapted to detect and quantitate SEB in biologic materials. The assay, which has a sensitivity level of about 8 ng of toxin/ml, employs ^{125}I-SEB as the radio-labeled antigen.

Publications:

None.

LITERATURE CITED

1. Greenwood, F. C., W. M. Hunter, and J. S. Glover. 1963. The preparation of ^{131}I-labeled human growth hormone of high specific radioactivity. Biochem. J. 89:114-123.

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AUTHOR INDEX

APPENDIX A

U. S. ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES GUEST LECTURE SERIES

DATE	GUEST LECTURER	TITLE OF PRESENTATION
17 Sep 70	Dr. Tov Omland Director of the Norwegian Defense Microbiological Laboratory Oslo, Norway	Infectious Disease Problems and Research in the Scandinavian Countries.
8 Oct 70	Dr. Robert I. Gregerman Chief, Endocrine Section National Institute of Child Health and Development, NIH Baltimore City Hospital Baltimore, Maryland	The Renin-Angiotensin System.
24 Nov 70	Dr. Walter Mertz Chief, Vitamin and Mineral Nutrition Laboratory U.S. Department of Agriculture Research Service Beltsville, Maryland	Essential Trace Metals - The Chromium Story.
17 Dec 70	Dr. John R. McCoy Professor of Comparative Pathology Rutgers University Medical School Brunswick, New Jersey	The Veterinarian's Role in Biomedical Research and the Influence of Laboratory Animal Welfare Legislation.
18 Feb 71	Dr. Fred L. Soper Special Consultant Office of International Health U.S. Public Health Service Department of Health, Education and Welfare Chevy Chase, Maryland	International Control of Communicable Disease.
18 Mar 71	Dr. William B. Greenough, III Chief, Infectious Diseases Div The Johns Hopkins Hospital Baltimore, Maryland	Cholera Enterotoxin: Key to Pathogenesis and Prevention of the Disease.
22 Apr 71	Lt Colonel Phillip K. Russell, MC Director, Division of Communicable Disease and Immunology Walter Reed Army Institute of Res Washington, D. C.	Pathogenesis of Dengue Virus Shock Syndrome.

APPENDIX B

U. S. ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES
PROFESSIONAL STAFF MEETINGS

DATE	LECTURER	TITLE OF PRESENTATION
18 Sep 70	Captain William S. Steinhart, MSC Physical Sciences Division	Liver Chromatin Template Activity in Pneumococcal Infection in Mice.
	Major Robert H. Fiser, Jr., MC Physical Sciences Division	Metabolic Fuel Interrelationships in the Host During Infection.
	Dr. Robert W. Wannemacher, Jr. Physical Sciences Division	Free Amino Acid Changes in the Host During Infection.
16 Oct 70	Captain Matthew J. Van Zwieten, VC Bacteriology Division	The Cellular Biology of Exogenous Proteins. Part I.
	Captain Peter G. Canonico, MSC Bacteriology Division	The Cellular Biology of Exogenous Proteins. Part II.
	Mrs. Mary H. Wilkie Bacteriology Division	Immune Responses Characterized by Immunoglobulin Classes and Serological Responses.
	Major Joseph Kaplan, MC Bacteriology Division	Studies of Cellular Immunity.
4 Dec 70	Major George W. Jordan, MC Virology Division	Production, Characterization and Bioassay of Chick and Mouse Interferons.
	Major David M. Robinson, VC Virology Division	Studies on the M-44 Strain of <u>Coxiella burneti</u>.
	Captain Neil H. Levitt, MSC Virology Division	Rapid Detection of Antibody o Viral AG by Cellulose Acetate Electrophoresis.
29 Jan 71	Major Frederick R. DeRubertis, MC Medical Division	Evidence for Enhanced Cellular Thyroxine-binding <u>in vivo</u> in Pneumococcal Infection.
	Captain Robin T. Vollmer, MC Medical Division	Automated Immunization Record System.

DATE	LECTURER	TITLE OF PRESENTATION
29 Jan 71	Lt Colonel Peter J. Bartelloni, MC Chief, Medical Division	Studies With Adenovirus Vaccine, Live, Oral, Type 21. Studies in Man With Chikungunya Vaccine.
26 Feb 71	Captain Douglas W. Mason, VC Animal Assessment Division	Effects of Acute Irradiation on the Response of Mice to TC-83.
	Captain Donald E. Kahn, VC Animal Assessment Division	The Development of a Radio-immunoassay for the Detection of Staphylococcal Enterotoxin B.
	Captain Joseph C. Denniston, VC Animal Assessment Division	Effects of Acute Infection on Cholesterogenesis in the Rhesus Monkey.
	Lt Colonel Richard O. Spertzel, VC Chief, Animal Assessment Division	Venezuelan Equine Encephalomyelitis in Central America.
26 Mar 71	Colonel Joseph F. Metzger, MC Chief, Pathology Division	*In vivo* Tritiation of SEB.
	Major William H. Adler, MC Pathology Division	Interaction of SEB with Lymphocytes.
	Lt Colonel William S. Collins, II MSC Pathology Division	A Solid Phase Radioimmunological Assay Procedure for the Determination of SEB.
	Major Hilary Evans, MC Pathology Division	Electron Microscopy of the VEE Virus.
	Captain Garrett S. Dill, Jr., VC Pathology Division	The Pathology of Trinidad Strain of VEE in Mice.
30 Apr 71	Lt Colonel Dan C. Cavanaugh, MSC Dept ent of Bacteriology Wa eed Army Institute of Rsch	The Use of Sentinel Animals for the Detection and Evaluation of Plague Foci.
	Mr. Daniel N. Harrison Microbiology Division	Serological Response of Rhesus Monkeys to Immunization and Infection with *Pasteurella pestis*.
	Colonel John D. Marshall, Jr., MSC Chief, Microbiology Division	Serological Studies of Individuals Immunized with Killed Plague Vaccine.

DATE	LECTURER	TITLE OF PRESENTATION
21 May 71	Captain Robin T. Vollmer, MC Moderator Physical Sciences Division	Panel discussion: Computer Uses in USAMRIID.
	Major Robert H. Fiser, Jr., MC Physical Sciences Division	
	Major George W. Jordan, MC Virology Division	
	Dr. Robert W. Wannemacher, Jr. Physical Sciences Division	
	SP4 Darrell A. Leonhardt Physical Sciences Division	

APPENDIX C

U. S. ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES
FORMAL PRESENTATIONS AND BRIEFINGS

Date and Group or Individual	Individual(s) Participating	Subject
16 Jul 70 Seminar to Staff of Environmental Services Branch, National Institutes of Health, Bethesda, Maryland	Colonel John D. Marshall, Jr., MSC	Environmental Health.
20 Jul-3 Aug 70 Government of Costa Rica; Middle America Research Unit, Balboa Heights, Canal Zone	Captain Donald E. Kahn, VC	In response to request through diplomatic channels, USAMRIID provided technical assistance in combating VEE epizootic in Central America.
28 Jul-12 Aug 70 Government of Honduras	Lt Colonel Robert W. McKinney, MSC	In response to request through diplomatic channels, USAMRIID provided technical assistance in use of VEE vaccine to control outbreak of VEE in Central America.
29 Jul-11 Aug 70 Government of Mexico, States of Chiapas and Oaxaca, and Mexico City	Lt Colonel Richard O. Spertzel, VC	In response to request through diplomatic channels, USAMRIID provided technical assistance in use of VEE vaccine to control an epizootic of VEE in Mexico.
30 Jul 70 Commander Noel L. Freeman, USN Commanding Officer, U.S. Naval Unit, Fort Detrick	Colonel Dan Crozier, MC	Orientation of USAMRIID research activities and tour of Phase I of new medical facility.

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Date and Group or Individual	Individual(s) Participating	Subject
5 Aug 70 Ms Jill Cintoli Mr. John Louigi Poli RAI Corporation, Italian TV New York City, New York	Colonel Dan Crozier, MC Lt Colonel Robert W. McKinney, MSC	Interview for TV program and visit to laboratories.
11 Aug 70 Brigadier General Louis J. Hackett, Jr. MC, Director, Plans, Supply and Operations. Office of The Surgeon General, DA; Colonels Robert Terrigan, Robert L. Krivulka, Harold Heady.	Colonel Dan Crozier, MC	Briefing on USAMRIID research facilities; and tour of post research facilities.
19 Aug 70 U.S.-Japan Joint Panel on Toxic Microorganisms, Bear River Research Station, Brigham City, Utah	Lt Colonel Harry G. Dangerfield, MC	Staphylococcal Enterointoxication Studies.
19 Aug 70 Dr. Claude Bradish Microbiological Research Establishment, Porton, England	Colonel Dan Crozier, MC	Review of USAMRIID research program and visit to Phase 1 of new medical facility.
24 Aug 70 Colonel William T. Fisher Commanding Officer, USA Regional Dental Activity, Walter Reed Army Medical Center, Washington, D.C.	Colonel Dan Crozier, MC	Tour of USAMRIID and post research facilities.
24 Aug 70 Dr. McRae, Member of The Presidential Scientific Advisory Committee, Office of Science and Technology, Washington, D.C.	Colonel Dan Crozier, MC	Presentation of future research program of USAMRIID in transition of Fort Detrick.

Date and Group or Individual	Individual(s) Participating	Subject
27 Aug-13 Sep 70 Government of Costa Rica, provinces of Guanacaste and Puntarenas.	Lt Colonel Richard O. Spertzel, VC	In response to request through diplomatic channels, USAMRIID provided technical assistance in use of VEE vaccine to control an epizootic of VEE.
8 Sep 70 Dr. F. J. Mulhern and Staff U.S. Department of Agriculture Washington, D.C.	Colonel Dan Crozier, MC	Participant in conference on VEE.
15 Sep 70 Dr. Robert Cook Microbiological Research Establishment, Porton, England	Colonel Dan Crozier, MC	Briefing on USAMRIID research program and visit to Phase I of new medical facility.
15-17 Sep 70 Colloquium on Selected Feline Infectious Diseases, Cornell University, Ithica, N.Y.	Captain Donald E. Kahn, VC	Respiratory Infection Complex, Epidemiological Studies.
17-22 Sep 70 Dr. Tov Omland Director, Norwegian Defence Microbiological Laboratory, Oslo, Norway	Colonel Dan Crozier, MC, and Staff	Briefing and discussions of USAMRIID research program; tour of all unit facilities.
21 Sep 70 U.S. Department of Agriculture Symposium for E. J. Underwood, Beltsville, Maryland	Dr. William R. Beisel, M.D.	Acute Changes in Trace Metal during Infection.

Date and Group or Individual	Individual(s) Participating	Subject
24 Sep 70 Annual Short Course: Pathology of Laboratory Animals, Armed Forces Institute of Pathology, Washington, D.C.	Lt Colonel James L. Stookey, VC	Systemic Mycoses.
		Iatrogenic Lesions of Laboratory Animals.
24 Sep 70 Annual Meeting of Commission on Epidemiological Survey, Armed Forces Epidemiological Board, Washington, D.C.	Colonel Dan Crozier, MC	Introductory Remarks.
	Lt Colonel Kenneth A. Woeber, MC	Infection and Thyroid Hormone Economy.
	Dr. Robert S. Pekarek	Trace Metals and Infection.
	Dr. Robert W. Wannemacher, Jr.	Amino Acid Metabolism and Infection.
	Major Robert H. Fiser, Jr., MC	Lipid Metabolism and Infection.
	Captain Michael C. Powanda, MSC	Tryptophan Metabolism and Infection.
	Captain William L. Steinhart, MSC	Nucleic Acid Metabolism and Infection.
	Major Gordon L. Bilbrey, MC	Renal Function and Infectious Illness.
	Dr. Anne Buzzell	Pathogenesis and Cellular Membranes.
	Captain Peter G. Canonico, MSC	Cellular Biology of Exogenous Proteins.
	Captain Neil H. Levitt, MSC	Detection of Virus by Immuno-electrophoresis.
	Captain Joseph Kaplan, MC	Studies of Cellular Immunity.

Date and Group or Individual	Individual(s) Participating	Subject
24 Sep 70 Annual Meeting of Commission on Epidemiological Survey, Armed Forces Epidemiological Board, Washington, D.C.	Captain William H. Habig, MSC	Cytochromes of *P. pestis*.
	Colonel John D. Marshall, Jr., MSC	Plague Vaccine Program.
	Major David M. Robinson, VC	Status of M-44 Q Fever Vaccine Studies.
	Lt Colonel Peter J. Bartelloni, MC	Recent Arbovirus Vaccine Studies in Man.
25 Sep 70 Continuation of Meeting of CES	Dr. William R. Beisel, MD	Infectious Illness and Performance.
	Captain William J. Caspary, MSC	Use of ESR in Infectious Disease Research.
	Colonel Joseph F. Metzger, MC	Pathology Division Studies.
	Captain Joseph C. Denniston, Jr., VC	Hypersensitivity Reactions to SEB.
	Lt Colonel Richard O. Spertzel, VC	Placental Transmission of Attenuated VEE Virus (TC-83).
4 Oct 70 Plague Seminar Fort Collins, Colorado	Colonel John D. Marshall, Jr., MSC	Study of Plague in the United States.
20 Oct 70 International Plague Seminar Saigon, Viet Nam	Colonel John D. Marshall, Jr., MSC	Use of Sentinel Animals in the Epidemiology of Plague.
21 Oct 70 74th Annual Meeting, U.S. Animal Health Association, Philadelphia, Pa.	Lt Colonel Richard O. Spertzel, VC	VEE, A Disease on the Move.

Date and Group or Individual	Individual(s) Participating	Subject
21 Oct 70 Interagency Meeting on International Health Activities, Washington, D.C.	Lt Colonel Robert W. McKinney, MSC	VEE in Central America.
26 Oct 70 Brigadier General Richard R. Taylor, MC Commanding General U.S. Army Medical Research and Development Command, Washington, D.C.	Colonel Dan Crozier, MC and Staff	Orientation briefing and tour of all USAMRIID facilities.
30 Oct 70 Symposium "Perspectives in Infectious Diseases" University of Michigan Ann Arbor, Michigan	Dr. William R. Beisel, M.D.	Metabolic Responses of the Host Tissues and their Role during Infection.
30-31 Oct 70 Meeting of Commission on Immunization Armed Forces Epidemiological Board Walter Reed Army Institute of Research	Colonel Dan Crozier, MC	Participant in meeting of the commission.
2-3 Nov 70 Dr. W. A. Snowdon, Dr. D. W. Howes, Messrs. J. V. Dunn, K. J. Walters, L. F. S. May Design Group from Commonwealth Scientific and Industrial Research Organization, Victoria, Australia	Colonel Dan Crozier, MC Dr. William R. Beisel, M.D. Lt Colonel Robert W. McKinney, MSC Lt Jack W. Downing, MSC	Discussion of laboratory design and tour of Phase I of new medical facility.
3 Nov 70 Naval Medical School Students National Naval Medical Center Bethesda, Md.	Dr. William R. Beisel, M.D.	Pre-clinical Laboratory Changes in Infectious Diseases.

Date and Group or Individual	Individual(s) Participating	Subject
9-10 Nov 70 University of Maryland School of Medicine, Baltimore, Md.	Dr. William R. Beisel, M.D.	Participant as a visiting professor.
12 Nov 70 Talbot County Medical Society Easton, Md.	Lt Colonel Robert W. McKinney, MSC	VEE in Central America.
12-13 Nov 70 Meeting of Commission on Rickettsial Diseases, Armed Forces Epidemiological Board, Walter Reed Army Institute of Research	Colonel Dan Crozier, MC Lt Colonel Robert W. McKinney, MSC Dr. Francis E. Cole, Jr.	Participants in meeting of the commission.
	Major David M. Robinson, VC	Status of M-44 Q Fever Vaccine Studies.
14-23 Nov 70 Meeting of Editorial Committee, Medical Defense Aspects of NBC Operations, NATO London, England	Colonel Dan Crozier, MC	Participant in meeting of the Editorial Committee on the NATO Handbook.
20-22 Nov 70 Four State Regional Meeting American College of Physicians Williamsburg, Virginia	Major Robert H. Fiser, Jr., MC	Lipid Metabolism and Acute Infection.
4-5 Dec 70 National Research Council, National Academy of Sciences Workshops on Zinc in Human Nutrition, Washington, D.C.	Dr. William R. Beisel, M.D.	Normal Serum Zinc Values in Young Adult Males. Altered Zinc Metabolism during Infection.

Date and Group or Individual	Individual(s) Participating	Subject
9-11 Dec 70 Annual Meeting of American College of Neuropsychopharmacology San Juan, Puerto Rica	Dr. William R. Beisel, M.D.	Influence of Acute Infection on the Metabolism of Zinc and other Trace Elements.
12-13 Jan 71 Annual Command Visit: Colonels Richard F. Barquist, MC, Thomas G. Murnane, VC, Robert L. Krivulka, MSC, Samuel L. Crook,MSC, Lt Colonels Donald W. Sample, MC, Milferd T. Guibor, MSC, Jules B. Lloyd, JAGC, Dallas P. Wright, MSC; all of USA Medical Research and Development Command	Colonel Dan Crozier, MC, and Staff	Annual orientation briefing relative to overall mission and operation of USAMRIID.
15 Jan 71 Foreign Animal Diseases Seminar, National Animal Disease Laboratory, Ames, Iowa	Lt Colonel Richard O. Spertzel, VC	VEE-The Disease and the Recent Outbreaks in Central and South America.
15 Jan 71 Global Medicine Course Walter Reed Army Institute of Research	Colonel John D. Marshall, Jr., MSC	Plague.
28-30 Jan 71 Southern Section, American Federation for Clinical Research Meeting, New Orleans, Louisiana	Major Robert H. Fiser, Jr., MC	Host Lipid Response during Infectious Illnesses.
2 Feb 71 Medical Grand Rounds University of Maryland School of Medicine, Baltimore, Md.	Dr. William R. Beisel, M.D.	Recent Metabolic Findings Associated with Infectious Illness.

Date and Group or Individual	Individual(s) Participating	Subject
4-11 Feb 71 Briefing and Survey, Fort Lewis, Madigan General Hospital and McChord AFB, Tacoma, Washington	Colonel John D. Marshall, Jr., MSC	Plague Investigation.
16-18 Feb 71 Annual Meeting, Biophysical Society New Orleans, Louisiana	Captain William J. Caspary, MSC	Spin-labelled Polynucleotides.
		Radical Formation of Carcinogenic Hydrocarbons and their Reaction with Purine Nucleosides.
17-19 Feb 71 Armed Forces Epidemiological Board Meeting	Colonel Dan Crozier, MC Dr. William R. Beisel, M.D.	Present report of the Commission on Epidemiological Survey.
25 Feb 71 Colonel Charles Pixley, 1st Army Surgeon, Ft Meade, Md. and Brigadier General Easterday, Commanding General, 804 Hospital Cntr.	Colonel Dan Crozier, MC, and Staff	Briefing on mission and scope of activities of USAMRIID; tour of Phase I of new medical facility.
12-17 Apr 71 Annual Meeting of the Federation of American Societies for Experimental Biology, Chicago, Illinois	Captain Michael C. Powanda, MSC	Relationship between Tryptophan Oxygenase Activity and Hepatic Nicotinamide Adenine Dinucleotide Concentration.
	Major Joseph Kaplan, MC	Staphylococcal Enterotoxin B Induced Release of Macrophage Migration Inhibition Factor from Normal Lymphocytes.
	Major Robert H. Fiser, MC	Effects of Acute Infection on Cholesterogenesis in the Rhesus Monkey.

Date and Group or Individual	Individual(s) Participating	Subject
12-17 Apr 71 (continued) Annual Meeting of the Federation of American Societies for Experimental Biology, Chicago, Illinois	Major John H. Boucher, VC	Effective B-Adrenergic Receptor Blockade with Sotalol in the Absence of Myocardial Depression.
	Captain Peter G. Canonico, MSC	Lysosomal Processing of Exogenous Proteins.
	Captain William L. Steinhart, MSC	Role of the Chromatin Template during Protein Synthesis in Mouse Liver.
	Dr. Robert S. Pekarek	Further Characterization of the Endogenous Mediator(s) of Serum Zinc and Iron Depression during Infection and other Stresses.
19 Apr 71 University of Wisconsin Department of Nutrition and Pathology Madison, Wisconsin	Dr. Robert W. Wannemacher, Jr.	Relationship between Tissue Amino Acid Concentrations and Regulations of Protein Synthesis in Various Disease States.
23 Apr 71 Colonel E.D. McMeen, Drs. B. F. Trum, T. C. Jones, Louis Melendez, Ronald Hunt; New England Regional Primate Center, Boston, Massachusetts	Colonel Dan Crozier, MC	Briefing and tour of Phase I of new medical facility.
23 Apr 71 Workshop on Simian Pathology Armed Forces Institute of Pathology, Washington, D.C.	Lt Colonel James L. Stookey, VC	Eosinophilic Myositis in a Rhesus Monkey.

Date and Group or Individual	Individual(s) Participating	Subject
27 Apr 71 Conference on VEE, Agricultural Research Service U.S. Department of Agriculture Washington, D.C.	Colonel Dan Crozier, MC	VEE - DOD Vaccine Supply
	Lt Colonel Richard O. Spertzel, VC	History and Present Status - VEE in Mexico and Central America.
28 Apr 71 The Women's Club of the Fort Detrick Officers' Open Mess	Colonel Dan Crozier, MC	Welcome and Briefing on Mission of USAMRIID.
	Lt Jack W. Downing, Jr., MSC Mr. Roy C. Culler	Tour of Phase I of new medical facility.
29-30 Apr 71 National Academy of Sciences Committee on International Nutrition Washington, D.C.	Dr. William R. Beisel, M.D.	Interrelated Changes in Amino Acid, Carbohydrate, and Fat Metabolism during Acute Infections.
29 Apr-1 May 71 Society for Pediatric Research Atlantic City, New Jersey	Major Robert H. Fiser, MC	Gram Negative Septicemia Versus Endotoxemia: Differential Effects of Lipid Metabolism.
1 May 71 Annual Meeting of American Society of Clinical Nutrition, Atlantic City, New Jersey	Major Robert H.Fiser, MC	Sequential Changes in Plasma Lipid and Lipoproteins during Sandfly Fever.
5 May 71 Annual Meeting of American Society of Microbiology, Minneapolis, Minnesota	Major George W. Jordan, MC	Application of Probit Analysis to Interferon Plaque Reduction Data.
	Dr. Bruno J. Luscri	Preparation, Bioassay, and Partial Fractionation of an Efficient L Cell Interferon.

Date and Group or Individual	Individual(s) Participating	Subject
5 May 71 (continued) Annual Meeting of American Society of Microbiology Minneapolis, Minnesota	Dr. Richard H. Kenyon	Development of an Improved Vaccine for Rocky Mountain Spotted Fever.
10 May 71 Research Training Fellowship Group Walter Reed Army Institute of Rsch Washington, D.C.	Dr. William R. Beisel, M.D.	Introductory Remarks.
		Host Metabolic Responses to Infectious Diseases.
	Dr. Robert W. Wannemacher, Jr.	Host Amino Acid Changes.
	Captain Michael C. Powanda, MSC	Hepatic Enzyme Changes.
	Major Robert H. Fiser, MC	Host Lipid Changes.
	Dr. Robert S. Pekarek	Host Trace Metal Changes.
	Colonel Dan Crozier, MC	Tour of Phase I of new medical facility.
		Vaccine Testing in Man.
	Major David M. Robinson, VC	Vaccine Development.
13 May 71 Dr. James Keppie Dr. Jack Melling Microbiological Research Establishment, Porton, England	Colonel Dan Crozier, MC Colonel Joseph F. Metzger, MC Dr. Virginia G. McGann	Briefing on USAMRIID Research Activities of mutual interest; tour of Phase I of new medical facility.

Date and Group or Individual	Individual(s) Participating	Subject
13-14 May 71 Computer Team from USA Medical Research and Development Command, Captain Roger Moore, MSC, and Mr. John R. Bradley (contract representative with Computer Sciences Corp., Falls Church, Virginia).	Colonel Dan Crozier, MC Dr. William R. Beisel, M.D. Captain Robin T. Vollmer, MC all division chiefs.	Participants in study for data automation requirements of USAMRIID.
18 May 71 Global Medicine Course Walter Reed Army Institute of Research, Washington, D.C.	Colonel John D. Marshall, Jr., MSC	Plague.
19-21 May 71 Meeting of the Armed Forces Epidemiological Board	Colonel Dan Crozier, MC	Present report of the Commission on Epidemiological Survey to the Board.
20-21 May 71 21st Annual Southwestern Conference on Diseases in Nature Transmissible to Man, College of Veterinary Medicine, Texas A and M University, College Station, Texas	Lt Colonel Richard O. Spertzel, VC	Current Status of Live Attenuated VEE Virus Vaccine -- Its Use in Central America and Mexico.
27 May 71 Infectious Disease Division Seminar University of Maryland School of Medicine, Baltimore, Md.	Dr. Robert W. Wannemacher, Jr.	Sequential Changes in Individual Serum, Amino Acids, Enzymes, and Trace Metals following Experimentally Induced Typhoid Fever in Man.

Date and Group or Individual	Individual(s) Participating	Subject
4-20 June 71 NATO Meeting of Editorial Committee, Medical Defense Aspects of NBC Operations, and Panel of Experts of The Technical Cooperation Program, Oslo, Norway	Colonel Dan Crozier, MC	Participant in meeting of the Editorial Committee on NATO Handbook; participant in TTCP Panel of Experts' Meeting as U.S. Representative on Medical Defense.
25 June 71 Foreign Animal Diseases Seminar, National Animal Disease Laboratory, Ames, Iowa	Lt Colonel Richard O. Spertzel, VC	Venezuelan Equine Encephalitis.

APPENDIX D

PUBLICATIONS OF U. S. ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES

FISCAL YEAR 1971

1. Abeles, F. B., R. P. Bosshart, L. E. Forrence, and W. H. Habig. 1971. Preparation and purification of glucanase and chitinase from bean leaves. Plant Physiol. 47:129-134.

2. Bartelloni, P. J., R. W. McKinney, F. M. Calia, H. H. Ramsburg, and F. E. Cole, Jr. 1971. Inactivated Western equine encephalomyelitis vaccine propagated in chick embryo cell culture. Clinical and serological evaluation in man. Amer. J. Trop. Med. Hyg. 20:146-149.

3. Beisel, W. R., and D. Crozier. 1970. USAMRIID seeks to develop therapy for biological agents. Army Res. Dev. Newsmagazine 11(7):68-69.

4. Beisel, W. R., and R. H. Fiser, Jr. 1970. Lipid metabolism during infectious illness. Amer. J. Clin. Nutr. 23:1069-1079.

5. Beisel, W. R., and R. S. Pekarek. 1971. Acute stress and trace element metabolism. Internat. Rev. Neurobiol., In press.

6. Bellanti, J. A., M. C. Yang, R. I. Krasner, P. J. Bartelloni, and W. R. Beisel. 1971. Studies of leukocyte function in the human during experimental sandfly fever virus infection. Fed. Proc. 20:396 (abstract).

7. Bilbrey, G. L., and W. R. Beisel. 1970. Depression of free water clearance (C_{H_2O}) during pneumococcal bacteremia. Program, Amer. Society Nephrology, 23-24 November, p. 8 (abstract).

8. Boucher, J. H., W. H. Zech, and A. L. Stagg. 1971. Effective β-adrenergic receptor blockade with Sotalol in the absence of myocardial depression. Fed. Proc. 30:228 (abstract).

9. Burghen, G. A., W. R. Beisel, and P. J. Bartelloni. 1970. Influences of chloramphenicol administration on whole blood amino acid in man. Clin. Med. 77:26-29.

10. Burghen, G. A., W. R. Beisel, J. S. Walker, R. M. Nims, D. L. Huxsoll, and P. K. Hildebrandt. 1971. Development of hypergamma-globulinemia in tropical canine pancytopenia. Amer. J. Vet. Res. 32: 749-756.

11. Canonico, P. G., M. Van Zwieten, and R. S. Pinkerton. 1971. Lysosomal processing of exogenous proteins. Fed. Proc. 30:6[illegible]0 (abstract).

12. Canonico, P. G., and M. J. Van Zwieten. 1971. Swelling of rabbit liver mitochondria induced by staphylococcal enterotoxin B. J. Infect. Dis., In press.

13. Caspary, W. J., B. I. Cohen, S. A. Lesko, and P.O.P. Ts'o. 1971. Radical formation of carcinogenic hydrocarbons and their reaction with purine nucleosides. Biophys. Soc. Abstracts 11:300a (abstract).

14. Caspary, W. J., L. M. Stempel, and P. Ts'o. 1971. Spin-labelled polynucleotides. Biophys. Soc. Abstracts 11:23a (abstract).

15. Chapple, III, F. E., J. M. Crosbie, and B. E. Reisberg. 1971. Surgical technique for cross-circulation of rhesus monkeys. Lab. Anim. Sci., In press.

16. Cole, Jr., F. E., and R. W. McKinney. 1971. Cross-protection in hamsters immunized with group A arbovirus vaccines. Infect. Immun., In press.

17. Craig, C. P., S. J. Normann, V. McGann, and W. S. Irvin. 1971. Chemotactic activity generated by staphylococcal enterotoxin B. Infect. Immun. 3:94-99.

18. Crozier, D. 1971. The physician and biological warfare. New Eng. J. Med. 284:1008-1011.

19. Dangerfield, H. G. 1971. Tularemia. In H. F. Conn (ed.) Current diagnosis, W. B. Saunders, Philadelphia, In press.

20. DeRubertis, F. R., and K. A. Woeber. 1971. Evidence for enhanced cellular thyroxine-binding in vivo in pneumococcal infection. Clin. Res. XIX:371 (abstract).

21. Feigin, R. D., W. R. Beisel, and R. W. Wannemacher, Jr. 1971. Rhythmicity of plasma amino acids and its relationship to dietary intake. Amer. J. Clin. Nutr. 24:329-341.

22. Fiser, R. H., J. C. Denniston, and W. R. Beisel. 1971. Gram negative septicemia versus endotoxemia: Differential effects of lipid metabolism. Program, Soc. Pediatric Res., Atlantic City, N. J., 29 April-1 May 1971, p. 235.

23. Fiser, R. H., J. C. Denniston, R. B. Rindsig, and W. R. Beisel. 1971. Effects of acute infection on cholesterogenesis in the rhesus monkey. Fed. Proc. 30:248 (abstract).

24. Fiser, R. H., R. S. Lees, W. R. Beisel, and P. J. Bartelloni. 1971. Sequential changes in plasma lipids during sandfly fever. Program, Amer. Soc. Clin. Nutr., Atlantic City, N. J., 1 May 1971, p. 3.

25. Fiser, R. H., J. C. Denniston, and W. R. Beisel. 1971. Host lipid response during infectious illnesses. Clin. Res. XIX:45 (abstract).

26. Habig, W., B. W. Hudson, J. D. Marshall, Jr., D. C. Cavanaugh, and J. H. Rust, Jr. 1971. Evidence for molecular heterogeneity of the specific antigen (fraction-I) of Pasteurella pestis. Infect. Immun. 3: 498-499.

27. Harrison, V. R., K. E. Eckels, P. J. Bartelloni, and C. Hampton. 1971. The production and evaluation of a formalin-killed Chikungunya vaccine. J. Immun., In press.

28. Huxsoll, D. L., P. K. Hildebrandt, R. M. Nims, and J. S. Walker. 1970. Tropical canine pancytopenia. J. Amer. Vet. Med. Ass. 157:1627-1632.

29. Jordan, G. W. 1971. Application of probit analysis to interferon plaque reduction data. Bact. Proc. 1971, p. 214.

30. Kahn, D. E., and T. E. Walton, Jr. 1971. Epizootiology of feline respiratory infections. J. Amer. Vet. Med. Ass. 158:955-959.

31. Kaplan, J., 1971. Staphylococcal enterotoxin B induced release of macrophage migration inhibition factor from normal lymphocytes. Fed. Proc. 30:647 (abstract).

32. Kenyon, R. H., W. M. Acree, F. W. Melchior, Jr., and G. G. Wright. 1971. Development of an improved vaccine for Rocky Mountain spotted fever. Bact. Proc. 1971, p. 85.

33. Kirkland, B. B., and J. H. Cranmore. 1970. Specific immuno-suppression effected with a factor in seromucoid extract. Transplantation 10:436-438.

34. Marshall, Jr., J. D., and D. C. Cavanaugh. 1970. Pasteurella, Chapter 20, p. 205 to 209. In J. E. Blair, E. H. Lennette, and J. P. Truant (ed.) Manual of Clinical Microbiology, American Society for Microbiology, Bethesda.

35. Pekarek, R. S., and W. R. Beisel. 1971. Further characterization of the endogenous mediator(s) of serum zinc and iron depression during infection and other stresses. Fed. Proc. 30:643 (abstract).

36. Pekarek, R. S. 1971. Effect of synthetic double-stranded RNA on serum metals in the rat, rabbit, and monkey. Proc. Soc. Exp. Biol. Med. 136:584-587.

37. Pekarek, R. S., G. A. Burghen, P. J. Bartelloni, F. M. Calia, K. A. Bostian, and W. R. Beisel. 1970. The effect of live attenuated Venezuelan equine encephalomyelitis virus vaccine on serum iron, zinc, and copper concentrations in man. J. Lab. Clin. Med. 76:293-303.

38. Powanda, M., and R. W. Wannemacher, Jr. 1970. Evidence for a linear correlation between the level of dietary tryptophan and hepatic NAD concentration and for a systematic variation in tissue NAD concentration in the mouse and the rat. J. Nutr. 100:1471-1478.

39. Powanda, M. C., and R. W. Wannemacher, Jr. 1971. Relationship between tryptophan oxygenase activity and hepatic nicotimide adenine dinucleotide concentration. Fed. Proc. 30:232 (abstract).

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45. Spertzel, R. O., and R. W. McKinney. 1971. Venezuelan equine encephalomyelitis in Central America and Mexico. Amer. J. Trop. Med. Hyg., In press.

46. Staab, E. V., S. J. Normann, and C. P. Craig. 1970. Alterations in reticuloendothelial function by infection with attenuated Venezuelan equine encephalitis (VEE) virus. J. Reticuloendothel. Soc. 8:342-348.

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57. Winnacker, J. L. 1971. Augmented growth hormone secretion in acute infection -- A specific response. Clin. Res. XIX:469 (abstract).

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